

To balance or not to balance budget?

A proposed amendment to the US Constitution requiring a balanced budget could cause great harm to fundamental research as well as to other social programmes on which the future of the United States now rests.

THE notion that the US government should spend no more than it receives in taxes and other income is perennially beguiling. It accords with the supposedly frugal housekeeping habits of the Founding Fathers. It would make the US federal deficit vanish at a stroke. And, by specifying how fiscal shortfalls should be bridged, it would rid members of Congress of the need for unpopular decisions on spending programmes. What can be wrong with such a recipe? Plenty.

So much should be plain from the past two weeks of debate in the US Senate. The present version of the proposition would eliminate the deficit and balance the federal budget by 2001. Its chief sponsor and advocate is Senator Paul Simon (Republican, Illinois), who is carrying the torch for an idea that was endorsed by the two previous (Republican) presidents, Ronald Reagan and George Bush. But nobody has said just which programmes would be eliminated and which taxes raised to bring federal revenues and expenditures into equilibrium.

That, no doubt, is why the Senate is making heavy weather of the plan. Those who favour the amendment talk about taxing the rich and cutting spending on instruments of war. Those opposed cite the end of Social Security as the elderly know it and predict that, were another flood to drown the midwest, as last summer, there would be no money to rebuild the nation's breadbasket. But all of that is mere hyperbole that begs the real issue, which is this: can the United States realistically contemplate vastly higher taxes on the middle class (not just the wealthy) and a genuine limit on what is called discretionary (as distinct from mandatory) spending on education, health care and scientific research?

The answer, plainly, is "NO". It is not in the national interest to tear down the very programmes that US society most needs in order to meet the intellectual and competitive challenges of the next century. Of course, there are ways to get rid of pork-barrel spending on useless projects that serve the special interests of members of Congress wanting to appeal to their hometown voters, but that would hardly be enough to balance the budget. And even the budget-balancers themselves have drafted provisions that would allow Congress to unbalance the budget again any time three-fifths of the members could be persuaded to vote an exception to the rules. But that exemption procedure is a recipe for a tough political battle on every case. The spoils would probably go to the most appealing cases: the victims of floods rather than physicists put out of work by reductions in federal support, for instance.

But the balanced-budget fervour is not lightly written off. The vote, which is about to come up in the Senate, is by no means certain. And there lies the rub. President Bill Clinton has made "investment" the buzzword of his administration. Selective spending on science and technology is indeed a genuine investment in future prosperity. But it is also one of the areas most vulnerable to a balanced budget amendment, no matter what its final permutations. The president asked for increases for science in his budget request for fiscal year 1995 (starting on 1 October 1994). But if a balanced budget is written into the Constitution, such increases would not be seen again. The research community, usually not exercised about spending issues in the abstract, should pay attention this time. □

Imanishi-Kari still in limbo

A finding is needed in a case that has been lingering since 1987. Justice delayed is justice denied.

IT is seven years since the US National Institutes of Health (NIH) began their investigation of alleged fraud by Thereza Imanishi-Kari, the Tufts University researcher who was one of the authors of a now notorious paper on immunoglobulin gene expression (*Cell* **45**, 247–259; 1986). The now-defunct NIH Office of Scientific Integrity (OSI) found her guilty of fabricating data and said so, in a draft document leaked to the press before Imanishi-Kari or her attorney could rebut the OSI's conclusions. Nothing much has happened since.

Of course, in 1989 the world all but found her guilty after a well-publicized hearing by Representative John Dingell (Democrat, Michigan), who called the Secret Service to give dramatic forensic evidence supposedly showing that Imanishi-Kari forged tapes from radioimmunoassays. She was not then and has not since been able to present experts who might challenge the Secret Service. The supposition of her guilt was also strengthened when the US federal attorney's office agreed to look into the case with an eye to criminal indictment. When the federal attorney dropped the case, the implication was that Imanishi-Kari was guilty, but that immunology is too intricate for her research to have been intelligible to a jury. Throughout this period, Imanishi-Kari has been denied the right to NIH grants and deprived of any meaningful opportunity to win tenure. And, for that, time is running out.



Yet the Office of Research Integrity (ORI), the successor agency to the NIH's OSI, which (being an independent body in the office of the Secretary of the Department of Health and Human Services, HHS) was supposed to tidy up the errors and excesses of the OSI, has so far failed to release its final report on the case, and this despite a recent appeal filed with ORI by Imanishi-Kari's attorney, who is one step short of suing the government. The attorney, Bruce Singal, says that justice delayed is justice denied. In this case, there can be no arguing with that position.

Nor can anybody deny that the whole question of fraud in science is now in turmoil in the United States. OSI began life believing that its investigations could be conducted as if they were research projects aimed at discovering the best approximation to the truth. In doing so, it paid scant attention to people's right to due process — to cross-examine witnesses, to be legally represented and so on. But people so accused can appeal to the HHS Departmental Appeals Board, when standard court rules on evidence apply. That is the board that last year dismissed findings of misconduct against Ramashwar Sharma of Cleveland and Mikulas Popovic, Robert Gallo's right-hand man in the development of a blood test for the AIDS virus. (OSI's successor, ORI, then promptly dropped a case against Gallo himself.) Imanishi-Kari will be entitled to an appeals board hearing if ORI finds against her. Given the stakes, that is only just.

The lessons to be drawn from this state of affairs are far from clear, but two shine through. First, ORI and its predecessor are not suited to the task for which they were created. At best, they are too cumbersome. ORI should be abolished. Responsibility for identifying misconduct and for imposing sanctions on those responsible should rest with research institutions unless criminal activity is involved. NIH and other public agencies should be concerned only to ensure that client-institutions are vigilant in this regard. The US National Academy of Sciences and Institute of Medicine have recently issued a paper on research integrity in anticipation of a spring conference. For ORI, there is a simple duty. Before it goes out of business, it should speedily say where it stands on Imanishi-Kari. Justice requires no less. □

Zeal earns influence

Philanthropist Mary Lasker was an extraordinary advocate for biomedical research.

CLICHES apart, the death of Mary Woodard Lasker at 93 on 21 February in New York brings to a close a unique era in US biomedical politics. The widow of Albert Lasker, an early success in advertising, Mary Lasker made a fortune of less than \$10 million to a biomedical research foundation whose influence far outmatched its size.

Among Lasker's skills was the use of her elegant Manhattan house to cultivate the rich and powerful in US politics, beginning in the 1940s with the chairmen of congressional committees, whose individual powers far outstripped those of their present-day successors. Best known for the Lasker

Awards in research and medicine, and for successfully parlaying a few subsequent Nobel prizes into the doctrine that the first precedes the second, Lasker's most significant and lasting achievement lay in her extraordinary influence over the purse-strings of the US Congress.

She made fast friends of representatives and senators who devoted their careers to fostering the growth of the National Institutes of Health. Nearly single-handedly she led the billion-dollar "War on Cancer" in 1971, perhaps in the knowledge that the money would be spent on fundamental research in cell biology as well as more classic clinical investigation of drugs and radiation therapy. She believed that research had but one purpose: the cure of disease. She had style and, with her single-minded determination to make sick people well, became an American institution. Times have changed. Politics are no longer as simple as they were. Nobody is likely to take her place. □

French language wars

The government in Paris is embarking on a misguided defence of the French language.

FRANCOPHILES everywhere will be alarmed by the bill on the use of the French language given outline approval last week by the French cabinet. If enacted, it will require, among others things, all scientific conferences in France to be held in French. *Nature*, which is planning two meetings in France this year (in October and December), has a vested interest in the bill's delay, but there is more than that to say.

M. Jacques Toubon, the minister of culture in Paris, is right to proclaim that there is a distinction between "international" and "universal"; French speakers are none the less international because of the language they speak, but would be diminished if compelled to speak some universal (and non-existent) Euroglot. Everybody, of course, agrees. Indeed, the rest of us would also be diminished if French were not the vivid language Toubon pleads for. But the question he raises goes deeper, and will not be put to rest by his hopes for machine translation: can French by compulsion serve the interests of France itself?

Sadly, the answer must be "NO". Two centuries of history have unfolded since the Revolution, and the world partly shaped by those great events is fashioned awkwardly for linguistic purists. The role of the English language in science (where it has only recently taken over from German) is a simple consequence of the growth of science in the United States since the Second World War and of the eagerness of researchers elsewhere to participate in and contribute to that endeavour. It is natural that those concerned should be resentful of this state of affairs; many have to learn a second language, and even then their contributions to the literature win less acclaim than those from familiar laboratories. But logic suggests the remedy is not to compel them to write or speak in their native languages, but to fund their own research enterprises so generously that the balance of linguistic advantage changes again. □

Global effort is launched to create taxonomic map of living organisms

San Francisco. Three scientific societies have joined together to urge the United States to take the leading role in launching an international project, estimated to cost \$3 billion a year, systematically to chart all the world's plants, animals and other living organisms.

Such a programme, they claim — which has been given the title Systematics Agenda 2000 — would effectively extend the proposed US National Biological Survey into a global enterprise.

Announcing details of the proposed initiative during the annual meeting of the American Association for the Advancement of Science in San Francisco last week, the biologists responsible for it said that the project would take 25 years to complete.

It would also require international commitment to a sixfold increase in the \$500 million being spent each year — most of it in the United States — on collecting and classifying species. But they said that at the present rate of progress, a decent inventory of the world's species will take 150 years to collate, and that pressures from human activities are now so great that the inventory must be completed much more quickly.

Systematics Agenda 2000 is being backed by the American Society of Plant Taxonomists, the Society of Systematic Biologists and the Willi Hennig Society, and has been put together with financial support from the

National Science Foundation.

Justifying its ambitious scope, Peter Raven, director of the Missouri Botanical Garden in St Louis, claims that the world will not thrive by building microchips, automobiles or the information superhighway. "It will succeed only if we manage microorganisms properly," says Raven.

The programme would have three main research elements. These would focus on the discovery of unknown species, on developing an improved understanding of the relationships between them and on developing computer networks and databases to store information about biodiversity.

Most of the work would be carried out in developing countries, which are thought to contain the widest range of biological species, and the programme proposals claim that various United Nations and other international agencies, could play a role.

But the plan remains vague about how these efforts should be funded. Its authors emphasize that they are proposing an international scientific collaboration, not an aid programme. But Raven, who is a member of the programme's steering group, argues that

even the poorest countries could benefit from investing money in preserving biodiversity. For example, he says, their ability to negotiate with international corporations keen to exploit local species will benefit greatly from "even two or three people" working on the topic.

The proposal cites projects such as those being conducted by Conabio in Mexico and the Instituto Nacional de Biodiversidad (INBio) in Costa Rica, as well as the US National Biological Survey, as models for a systematic approach to biological diversity.

Another biologist who has been involved in drawing up the proposed programme, David Wake of the University of California at Berkeley,

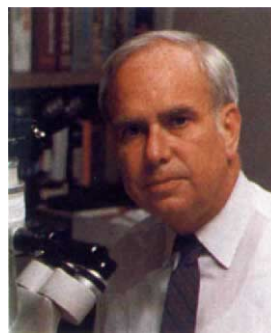
says the main need is for more information and knowledge. "When we spend money, we should do it in an intelligent way," says Wake, whose own interest is in the taxonomy of toads. He claims that governments only act to conserve "cuddly" species, such as the spotted owl, while those of greater scientific importance are allowed to become extinct.

Joel Cracraft of the American Museum of Natural History in New York, the chairman of the group that prepared the Agenda proposals, acknowledges that their publication is merely the first step in a long process of building international support.

The process will continue with symposia being planned in London and Paris in the spring, and another by the United Nations Educational, Scientific and Cultural Organisation (UNESCO) later in the year. The London meeting takes place at the Royal Society on 12 April, to be followed on 15 April by a meeting organized by the Société Française de Systematique in Paris.

Key members of the planning group will be attending the European meetings. "The idea is to publicise this initiative and advance the agenda," says Michael Claridge, professor of zoology at the University of Wales in Cardiff and one of the speakers at the London symposium. "We all agree is a marvellous idea and a very ambitious one."

But the backers of Systematics Agenda 2000 acknowledge that it may be difficult to find support outside the United States, where public awareness of the biodiversity issue is already high. Raven claims that many European countries are holding back from making any significant financial commitments, while Japan "is almost totally insensitive to biodiversity issues". **Colin MacIlwain**



Raven: 'we need to manage properly'.

US Congressmen in bid for medical funds

Washington. In an attempt to emphasize that support for medical research must go hand in hand with healthcare reform, two US Senators this week unveiled a legislative proposal to create a national fund for medical research within the US Treasury.

If enacted, the fund would be expected to provide an estimated US\$5 to \$6 billion annually in additional support for the US National Institutes of Health (NIH), at a time when discretionary spending within the government has been frozen for the next four years.

The fund would be financed from two sources. One would be a one per cent set-aside from all monthly health insurance premiums; the other a provision enabling the public to make voluntary contributions by marking a box on their annual federal income-tax returns.

Money allocated through the fund would not be earmarked for specific areas of research. Rather it would be distributed to NIH institutes and centres in proportion to the amount which each receives through the annual appropriations process. To ensure

that the fund supplements, rather than supplants, existing NIH appropriations, money would be made available through it in any one year only if Congress matches or exceeds the previous year's appropriation.

The proposal, which has bipartisan backing in the Senate, is being sponsored by Senators Tom Harkin (Democrat, Iowa), Mark Hatfield (Republican, Oregon), Nancy Kassebaum (Republican, Kansas) and Edward Kennedy (Democrat, Massachusetts). Similar legislation is expected to be introduced shortly in the House by Bill Coyne (Democrat, Pennsylvania).

"It is past time to expand the debate on healthcare," Harkin said on Monday. Until the importance of medical research receives serious emphasis, "every other step we take on healthcare reform is just like rearranging deckchairs on the *Titanic*."

A recent Louis Harris poll of more than 1,200 adults found that more than 70 per cent of those surveyed would be willing to pay US\$1 more per week in health insurance premiums if they knew it would go towards medical research. **Diane Gershon**

Top Russian institutes get reduction in power bills

Moscow. The Russian government has granted 50 research institutions a reprieve from the crippling electricity bills they have been facing as a result of recent inflation. The government has announced that these institutions will have to pay only half of the money they owe. But another 270 institutes that were originally scheduled for tariff reductions are being left to negotiate discounts individually with local officials of the electricity supply company.

Electricity bills have increased 15-fold since the beginning of 1993, but the science budget has only been increased by a factor of four in the same period. Some of these bills, issued by the company Unified Electric System, are comparable in size to the total state budget allocated to the institutes, and frequently exceed the funds available, as the money is paid in instalments.

According to Boris Saltykov, the Russian Minister of Science, the problem had resulted from the lack of legislation for "non-commercial organizations", which would allow research institutes to pay for energy at a reduced rate compared with commercial enterprises.

Most Russian institutions have been affected by inflated electricity bills. Despite severe cutbacks in electricity consumption, the Kurchatov Institute of Atomic Energy, for example, is in serious debt, and has had trouble paying scientists' salaries on time.

The Institute of High Energy Physics in Protvino can afford to switch on its accelerator for only two or three weeks each quarter. At the start of the year, the govern-

ment was late in paying the institute's grant, and scientists had to decide between turning on the accelerator and getting paid.

But the Joint Institute for Nuclear Research (JINR) in Dubna has not had the choice of turning off the equipment, as it is bound by international agreements. As a result, it is accumulating an enormous debt.

Last November, Vladimir Kadyshevsky, the director of the JINR, asked Yegor Gaidar, then first deputy premier, to resolve the problem (*Nature* 367, 208; 1994). An agreement was reached with the Ministry of Fuel and Energetics, and was supposed to be signed on 26 December.

Since then, however, the signing of the agreement has been postponed several times, and Gaidar's resignation in December led to speculation that the deal had fallen through. The Ministry of Fuel and Energetics is known to oppose the idea of reduced rates for research institutes, although it did not openly refuse to sign the agreement.

Saltykov's announcement that only half of the electricity bills will have to be paid will help the institutions concerned. But the remaining 270 institutes will have to find some other way to deal with the problem. One solution would be for these institutes to approach officials of the energy agencies directly, rather than using the government as an intermediary. The director of one such institute says they may be given equivalent reductions because local officials often resent central intervention, and are sympathetic to local debtors.

The directors of the institutes that have applied for reduced tariffs are watching the struggle between ministries not only with concern for their institutes but also to see how much influence Saltykov actually holds in the government. Some feel that not only the minister's reputation, but possibly his seat, is at stake.

Vladimir Pokrovsky

IMAGE
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**Saltykov: reputation
is at stake.**

V. Cherdantsev/TASS

Canada drops its plans to build K-meson facility

Quebec. In its first budget since winning last October's general election, Canada's Liberal government has scuttled the 'Kaon Factory' project, shifted the focus of the country's space programme away from the US space station towards domestic needs, and promised sustained support for its national research councils.

The demise of the Kaon project, designed for the mass production of K-mesons, will follow the withdrawal of C\$236 million (US\$184 million) promised by the former Progressive Conservative government. The controversial project was to have been an extension of TRIUMF (Tri-University Meson Facility) in British Columbia, and was intended to place Canada in the forefront of particle physics research.

It was Kim Campbell, defeated in the polls by Jean Chrétien in her bid for re-election as prime minister, who promised support for the project in 1991 when she was justice minister. At the time, she was the House of Commons representative for the province of British Columbia.

Her announcement drew immediate charges of political favouritism, as her government's own science advisory bodies — including the National Advisory Board for Science and Technology which Prime Minister Brian Mulroney chaired — had unanimously recommended against the project.

Janet Halliwell, then chairperson of the Science Council of Canada, said at the time that, given the advisory bodies' recommendation, the government's decision raised a question mark over their role. The Science Council was later dissolved by the Conservative government.

Canada's position on the space station remains uncertain. In a draft of the budget speech distributed to the press, Paul Martin, the finance minister, announced that Canada would "negotiate an orderly withdrawal". But after a direct telephone appeal from President Bill Clinton, he changed this to "an orderly reduction" in commitments to the programme.

The plan is to reduce the amount the previous government had promised, and to add a further C\$800 million for a new, long-term Canadian space programme that will emphasize applications perceived to be in Canada's best commercial and job-creating interests, such as remote sensing and telecommunications satellites.

Finally, apparently responding to concern about underfunding of the National Research Council (NRC), the government promised a funding increase for NRC's 1994-95 budget. It has also spared from cuts all the bodies that provide money for university research.

David Spurgeon

Kohl to chair German advisory council

Munich. As part of its campaign to improve cooperation between the scientific community, German industry and government policy-makers, the German cabinet last week decided to set up an advisory council for research, technology and innovation, to be chaired by the Chancellor, Helmut Kohl.

The council has the strong support of the Ministry of Research and Technology (BMFT), which will provide it with administrative support. In addition to Paul Krüger (CSU), the minister of research, its members will include the minister of education and the economics minister, as well as repre-

sentatives from science, industry, trade unions and the government.

The names of those appointed to the council are expected to be announced by the chancellor within the next two weeks, and the council has been asked to address issues of importance to Germany's economy.

Possible themes to be investigated include ways of encouraging the public's acceptance of new technologies, and how to improve the implementation of inter-ministerial decisions, for example by funding personnel exchanges between universities and industry.

Allison Abbott

Improved offer made to ex-GDR researchers

Berlin. The Humboldt University in Berlin has backed down from an attempt to give only 'second-class' contracts to 250 scientists funded under a programme to integrate east German scientists into universities in the new *Länder* following protests that the contracts were unfair.

In 1990, 2,000 top-rated scientists were transferred from the overstuffed research institutes of the former German Democratic Republic to the state-funded Wissenschaftler Integrations Programms (WIP), designed to help re-establish research in universities.

The DM600 million (US\$300 million) programme supported the scientists fully for two years up to last December, enabling them to find university posts. Further support is being provided during the first three years of their university employment, with the university paying an increasing proportion of their salary each year.

The original idea was that by 1996 all the WIP scientists would have permanent university posts. But the universities have resisted this, pointing out that since reunification they have fired thousands of their own staff, and have no desire to dismiss more in order to accommodate 'outsiders'.

It now looks as if only a handful of the scientists will have permanent positions when the state-subsidized period ends in 1996. In contrast, the number of short-term contracts issued to WIP scientists — 1,473 by universities and 207 by other institutes — has been much higher than expected.

But even here there have been problems. Almost all the universities in the new *Länder* offered the WIP scientists standard short-term contracts. But the Humboldt University, the former East Germany's most important university, introduced special clauses in the contracts which appeared to give its 250 WIP scientists a 'second-class' status.

For example, nearly all were placed on the lowest point of the university pay scale, irrespective of previous experience. They have been required to accept a six-month probation period, even though many have been running lecture courses for more than a year. And the contracts state explicitly that the WIP scientists will not be given permanent employment at the end of three years.

Several groups of scientists have complained about these conditions. "They are humiliating and unfair, and certainly against the spirit of the WIP programme," says one WIP scientist.

Detlev Ganten, director of the Max Delbrück Centre for Molecular Medicine — a national research centre in Berlin which took on a hundred WIP scientists in the programme's first two years — is among those who have declared their support. "I have a lot of sympathy for these people," he says. "Some have not been treated as they should have been."

But Ganten and others accept that Humboldt has itself been a victim of the times. Since reunification, the university has had to shed more than half of its staff,

and all professors have had to be reselected through open competition.

The prospect of taking on a large proportion of WIP scientists seems to have been the last straw. Ganten claims that "with more administrative experience and less nervousness, it could all have run more smoothly". Wieland Hampel, of Berlin's research ministry agrees.

But the university itself claims that it placed restrictions on the contracts for financial reasons. Although the federal government provides an average of DM100,000 a year for each scientist, the university says the money is not sufficient to cover all the costs involved. Nevertheless, following the protests about its policy the university has agreed in principle to review all the positions offered to WIP scientists, and make up any shortfalls in pay considered necessary.

Alison Abbott

India lifts curbs on foreign R&D

New Delhi. India has agreed to ease restrictions on foreign multinational companies wishing to set up research and development (R&D) facilities in the country, in line with commitments under the revised General Agreement on Tariffs and Trade (GATT) concluded in Geneva last December.

With the exception of research in "atomic energy and related matters", foreign companies are now free to establish R&D centres in which they have a controlling stake in almost all areas of science and engineering.

In the past, the government has been cautious about allowing foreign investment in R&D facilities. But given the present decline in public funding for research, combined with the reluctance of Indian companies to invest in their own research activities, the government has agreed to welcome foreign multinationals in the hope of stimulating the research base.

Foreign participation in applied science and technology will be allowed in a wide range of industries, including communications, electricity production, marine engineering, aeronautics engineering, construction and information technology.

The government will also allow multinationals to be majority equity holders in facilities for technical testing and analysis, in the hope that this will lead to an improvement in quality control systems and the general raising of technical standards.

Several multinational drug companies, including Astra of Sweden, have already established R&D centres in India. Until now, however, the government has given permission for these centres only on a case-by-case basis.

K. S. Jayaraman

Gene transfer patent is revoked

Munich. A patent granted in 1992 to the agrochemical and pharmaceutical company Ciba-Geigy on a technique for transferring genes directly into plant cells has been revoked by the European Patent Office (EPO) following a successful challenge by the Swiss company Sandoz and the German AgrEvo, jointly owned by Hoechst and Schering.

Ciba-Geigy had claimed broad patent protection for its technique, which would have covered all methods for direct gene transfer without the aid of a vector, including chemical treatment or electroporation. Although direct gene transfer is not widely used by plant genetic engineers, the breadth of the patent had caused concern, because of fears that it could restrict the freedom of plant breeding companies in the future.

But Sandoz and AgrEvo last week persuaded the EPO to revoke the patent, based on work carried out at Ciba-Geigy's Friedrich Miescher Institute in Basel, on



"Kein Patent auf Leben!" the EPO disagreed.

the grounds that some of the methodology had been published before 1984, when the application was submitted.

At the same time, a challenge filed by environmental groups was rejected by the EPO's 'opposition board'. This said it did not accept that patents on genetically manipulated organisms posed a "threat to public morality or public order", so they did not contravene the European Patent Convention as the groups had claimed. A. A.

French nuclear research goes back to basics

Paris. For the second time in less than a decade, France's Atomic Energy Commission (CEA) is changing direction. The move has been prompted by the need to justify its huge cost to the taxpayer — FF6.54 billion (US\$1 billion) annually for civil research alone — at a time when the expansion of nuclear power has virtually come to a halt in the country.

In the mid-1980s, as the research needs of the nuclear industry were beginning to diminish, the CEA sought to redefine its role by expanding its involvement in the industrial applications of its technology. Now it is retreating from direct participation in the development of new technologies, and focusing once again on long-term research underpinning areas such as nuclear technology, reprocessing and safety.

The shift will ease pressure on CEA's finances; research is cheaper than development. Furthermore, many argue that, because CEA is a public body, it should leave industrial ventures and short-term research to commercial companies.

CEA has already started to move in this direction. For example, it has transferred the manufacture of mixed uranium/plutonium (MOX) fuel to COGEMA, France's state-backed nuclear fuel company. Yannick d'Escatha, deputy chairman of the commission, says it will now increase its efforts to find companies to take over its work on other mature technologies, such as the production of integrated circuits, the dismantling of nuclear plants and industrial robotics.

In future, says d'Escatha, CEA will invest less of its own money in development projects, and instead encourage others to turn its research into new products. One consequence, says d'Escatha, is that CEA will have to involve companies in its research programmes at a much earlier stage than at present.

Another result of this change in direction is that CEA Industrie, the CEA's powerful industrial holding company, is likely to slim down into little more than a technology-transfer company. Last month, for example, it announced that it is giving up its majority shareholding in several high-technology companies; it also plans to reduce its stakes in other large companies, such as the nuclear engineering company Framatome, when these are privatized.

The 1994 budget for CEA shows the beginning of the shift back to medium- and long-term fundamental research. The budget for research on the separation and incineration of actinides and long-lived fission products is increased 20 per cent, to FF257 million; that of the use of fast breeder reactors as plutonium furnaces goes up 12 per cent to FF217 million; and studies on storage of waste will be boosted by 31 per cent, to FF134 million.



d'Escatha: looking outside the CEA.

There will also be increased funding for research on the next generation of pressurized water reactors (PWRs, up 27 per cent to FF172 million), and on the use of mixed oxide fuels (up 18 per cent to FF115 million). The largest items in the research budget remain dismantling (FF566 million), PWR safety (FF509 million) and laser enrichment of uranium (FF447 million).

In addition, the government has stated that CEA should support long-term research in non-nuclear areas of "scientific, economic or strategic importance" and of an international standard. This will allow the CEA to answer the long-standing criticism that proliferation of its research portfolio — for example, it now has research groups in climatology, particle physics, protein engineering, electronics and radiobiology — has taken place in an unplanned way.

Fields now earmarked for increased support, with the backing of both industry and government, include microelectronics and nanotechnology (up 3.6 per cent to FF375 million), photonics (up 4.5 per cent to FF157 million) and radiometry (up 10 per cent to FF61 million).

One problem facing CEA is adapting its workforce, which has many engineers, to the switch to research. In some fields, CEA has been retiring engineers early and recruiting scientists in their place. But it is being hampered by the overall staff cuts needed to pay for increases in research programmes; staff numbers have fallen from 23,000 to 18,000 over the past five years.

CEA is also having to find the money for a ten-year plan to boost its investment in big equipment. Responding to previous budget cuts, the commission had reduced such spending from around FF3.2 billion in 1987 to less than FF1.5 billion in 1992 to maintain spending in other areas. It will now increase by 18 per cent this year.

The overall research budget is growing again after repeated cuts over the past decade. Moreover, the demise of the European Fast Reactor programme is expected to save the CEA about FF200 million. The commission also intends to close the SATURN accelerator at Saclay near Paris.

Some observers claim that the reforms do not go far enough, suggesting, for example, that the commission should spend more on exploring new ideas for reprocessing. One senior official even argues that CEA should act as the international spokesperson for the French public on nuclear issues, and therefore needs to become completely independent of the nuclear industry. But that is likely to be too radical a step — even for France.

Declan Butler

Immigration law 'a barrier to collaboration'

Paris. The French government has been accused of dragging its feet on a promise to exempt foreign scientists from clauses in a new immigration law that would prevent them from being joined by their families for two years after their arrival in France.

The promise was made last summer by Charles Pasqua, the Minister of the Interior, after persuading two members of parliament to withdraw amendments that exempted invited researchers from the requirement being imposed on workers from outside the European Union and from the complex administrative procedures now required for visits of even a few days.

But seven months later, Pasqua's promised 'circulaire', which would adjust the way the law is enforced, remains bogged down between the several ministries involved.

Meanwhile, claims Edouard Brezin, the president of the Centre National de la Recherche Scientifique (CNRS), civil servants are "brutally enforcing" the law, and refusing to grant residence to families of visiting researchers. "It's absurd", says Brezin, who has championed the scientists' cause. "How can we invite researchers, and then

tell them they can't bring their families?"

Brezin has arranged with the Ministry of the Interior for the entry for several families. But he remains concerned about cases he is unaware of, as well as the impact of the law on potential visitors. "It will destroy our exchanges," he says.

Brezin says he is convinced that Pasqua supports the case for exempting scientists. But he complains that the government has kept him in the dark about the progress of the circular, and expresses concern about apparent resistance in some ministries.

François Fillon, the minister for higher education and research, has not been prominent in defending the scientists' cause. Although he officially expressed concern in a letter to Pasqua last June, his ministry was not represented at a meeting in July attended by Brezin and representatives from both the ministries of health and foreign affairs to discuss the circular.

A spokesman for the Ministry of the Interior says that the circular is "blocked" in another ministry. But he refused to comment either on what the circular says, or when it is likely to come into effect. **D. B.**

Conflict grows over breast cancer strategy

San Francisco. Last week's annual meeting of the American Association for the Advancement of Science (AAAS) became the site of the latest skirmish in an increasingly bitter conflict between US scientists working on the prevention and treatment of breast cancer and activists who claim that government-backed efforts in the field are misguided.

The clash provided further evidence of the extent to which, in the absence of scientific consensus on the causes of cancer, groups that feel more emphasis should be placed on rapidly selecting and eliminating environmental causes are determined to make themselves heard.

"We must not allow the scientific community to tell us that what we need is more research," says Samuel Epstein, a professor of medicine at the University of Illinois at Chicago, speaking at a panel session on breast cancer research policy in the US.

Epstein, who claims that sufficient data exist to justify the banning of a number of allegedly carcinogenic substances, used the occasion to launch an attack on the policies

of the National Cancer Institute (NCI), the main research funding body in the field, which is part of the National Institutes of Health.

But he and other session panel members were challenged by Mina Bissell, a biologist at Lawrence Berkeley Laboratory in California. Bissell said that Epstein had inadequate data to back up some of his claims, and — to cheers from half of the 300 people in the hall — criticized the AAAS for arranging a session which had ended up as "an attack on all of science".

"If you don't build your bridges with the help of scientists and engineers, you will fall into the ocean," Bissell told the meeting as she was crowded away from the microphone by the two co-chairs of the session, Janice Kirsch of the health care group Kaiser Permanente at Hayward, California and Nancy Evans of the lobby group Breast Cancer Action. Order was then briefly lost as supporters of the panellists, seated at the front of the hall, exchanged insults with the sceptical scientists at the back.

During these exchanges, Epstein said he

was not being critical of individual researchers, but argued that "the science community is held hostage" to the political process that directs its work. Earlier, he had delivered a barrage of criticism of the American Cancer Society (ACS), the NCI and other government agencies. He accused the NCI of spending only 3 per cent of its \$1.9 billion annual research budget on identifying "avoidable carcinogens", while pumping money into research on the effects of a fatty diet, evidence for which was "tenuous".

Epstein said that the expanding use of X-ray mammography to screen women for breast cancer in the US was "reckless if not criminal". He attacked as "flawed" a recent study by the ACS and the Food and Drug Administration (FDA) which concluded that there was "almost no connection" between hair dyes and cancer, and he proposed a link between breast cancer and the consumption of milk from BST-supplemented cows, due to allegedly high levels of insulin growth factor (IGF-1) in the milk.

The breast cancer session also included presentations linking the disease with medical X-rays, pesticides, postmenopausal hormone therapy and exposure to various chemicals that function as xenoestrogens.

Lobbies such as Breast Cancer Action and the Cancer Prevention Coalition, which Epstein chairs, say they have learned much from the AIDS activist movement, and their determination to be heard looks certain to raise the profile of public debate on cancer research policy.

Colin Macilwain

How a meeting of minds can spell trouble

Clashes of culture can undermine international joint research ventures, researchers from Ohio State University and Lehigh University told last week's meeting of the AAAS. Foreign research laboratories also suffer from a mismatch of values and perceptions fostered by different societies.

A study of 235 engineers and managers at 40 US-Japanese joint ventures showed that participants from the two countries disagreed on preferred ways of communicating, of improving production and of measuring performance, said Robert Mason, of the Center for the Management of Science and Technology at Case Western Reserve University in Ohio.

According to Japanese engineers, 'quality' meant the best that could be achieved at the moment, based on state-of-the-art knowledge, and 'effectiveness' was demonstrated by levels of creativity and innovation. US workers said high quality meant meeting specifications, and talking to customers was the best measure of effectiveness.

Mason said such disagreements often remain unspoken, and can lead to conflict. He recommended that participants in joint projects be trained to look for hidden assumptions, and to define their terms.

Joseph Cheng, associate professor of international business and management at Ohio State University, said companies can enhance the performance of an overseas laboratory or joint venture by designing an appropriate corporate culture. For example, a "technology transfer" laboratory organ-

ized to adapt a product for use in a foreign market would benefit from strong influence from corporate headquarters.

Cheng added that a laboratory director from the home office, who built up strong reporting relationships and made frequent visits to headquarters, would help strengthen the influence of the corporation. But a laboratory intended to develop products for local markets would operate better with strong local influence and less corporate attention.

Theodore Schlie, associate professor of technology management at Lehigh Univer-



sity in Pennsylvania, said communication between headquarters and the foreign laboratory was vital. Temporary foreign assignments, electronic communication and "meetings, meetings, meetings" all contributed to this goal.

Sally Lehrmann

Genome project 'has opened Pandora's box'

The Human Genome Project has raised ethical issues that no-one knows how to deal with, according to several speakers addressing a discussion on genetic research into sexual orientation during the AAAS meeting.

Dean Hamer of the NCI, who last July published results linking genetics and homosexuality that triggered intense debate, told the meeting that "in dealing with human sexuality, we have a lot more to fear from ignorance than from my work".

According to opinion polls, many parents, given the choice, would abort fetuses carrying a range of behavioural and physical characteristics. Hamer said that any form of genetic screening based on his work "would be wrong", but he did not specify how it could be prevented.

Donald Gabard, of the department of physical therapy at Chapman College, California, said the whole human genome project had "opened a Pandora's box" that neither researchers or doctors could be trusted to close.

C. M.

UK innovation centre fails to meet some expectations

London. As the government of British Prime Minister John Major looks to 'technology foresight' (see below) for ways of stimulating the commercial exploitation of science, rumblings of unease are being heard about a major initiative launched by the government of his predecessor, Mrs Margaret Thatcher, to achieve precisely this goal.

The Centre for Exploitation of Science and Technology (CEST) was set up in 1988 at the explicit recommendation of the government Advisory Committee on Applied Research and Development. It operates as a think tank jointly sponsored by government and industry, exploring the market potential for new science-based products.

But a confidential survey of companies represented on the council has found that about half of those interviewed would like to see a better return on their investment. All said they would have some difficulty in justifying renewing their subscription.

The survey was carried out by an outside consultant. It reports that most of the companies who support CEST are enthusiastic about the work it carries out, benefiting in particular from involvement in individual projects which had increased their contact with others in the field.

But one-third of 82 individuals contacted from among the council and working group members claimed that CEST was not strong enough politically, and 90 per cent said that they viewed CEST as "badly deficient in communications".

Companies belonging to CEST come from the top ranks of British industry, and include names such as Rolls Royce, Glaxo and ICI. Most currently pay a membership fee of about £58,000 a year, and additional support comes from the government. The Office of Science and Technology, for example, provides funding of £116,000 a year out of the science budget.

One of CEST's best known reports was one published three years ago putting the case for the creation of so-called Faraday centres, to provide a focus for interaction between the academic world and industry. The idea was enthusiastically endorsed by the Tory government shortly before the general election in 1992. However it was dropped after the election by the President of the Board of Trade, Michael Heseltine, on the grounds that new institutional structures were unnecessary.

Apart from this, however, CEST appears to have made less of an impact on major policy debates than some of its supporters had hoped. It also raised some eyebrows with its submission to the government during preparations for last year's science white paper (policy document). This suggested

that government funds for basic research be channelled through an independent Industrial Technology Council, and described the undirected use of public resources to support science and technology as "a luxury we cannot afford".

CEST has not grown as fast as it had hoped. Last year's annual accounts report an operating loss of £300,000 on turnover of £1.7 million; part of this was due to a dispute with the Inland Revenue over VAT payments, but CEST also admitted that income had not met expectations "due to the difficulty of finding new member companies". Since then, two of its member companies — British Telecom and Thorn/EMI — have resigned, increasing the financial pressure on the organization.

Bob Whelan, CEST's director who was formerly the marketing manager of the consulting firm P A Technology, denies that the organization is in difficulties. He declined to comment on the contents of the report, on the basis that its conclusions are confidential. But he acknowledged that the report identified "areas that are going well," as well as "some that we need to do work on."

Sir John Fairclough, the chairman of CEST and formerly Mrs Thatcher's chief scientific adviser, says that a working group had now been set up which was likely to suggest that CEST focus more on strategic issues. Such a move which could mean relatively less emphasis on practical topics of interest to only a few of its members.

According to one observer, CEST has suffered from ambiguity over whether it is primarily a government or a private sector initiative. Whether technology foresight will become vulnerable to the same charges remains to be seen.

David Dickson

Women 'should fill a quarter of top scientific posts'

London. Britain's Office of Science and Technology (OST) has been urged to set up a 'development unit' to oversee the interests of women scientists. The government has also been told that it and other employers should aim at seeing women occupy one quarter of all public appointments and senior positions in science, engineering and technology by the end of the decade.

Jean Balfour, the deputy chairman of the Women in Science, Engineering and Technology committee, which presented its recommendations to the government last week in a report entitled *The Rising Tide*, said a development unit in the OST would be a key focus for the government in this area.

The panel suggested that the unit should quantify the benefits of 'women-friendly' practices, such as the provision of crèches, flexible working hours and working for employers from home.

"This report is really a first step looking towards the future," said Balfour last week. "Women scientists and engineers have particular problems, as the period when their careers are at a crucial stage often coincides with the time that they are occupied with bringing up families."

But problems may take root much earlier, with fewer girls opting to continue with science subjects after completing the compulsory science syllabus at 16. The report points to the success of the Scottish system, under which pupils normally take up to five subjects in a more advanced examination at the age of 17.

In 1991 girls achieved almost half the total number of passes in mathematics and chemistry in the Scottish examinations, compared to 36 per cent and 41 per cent respectively in England.

Fiona Gammie

Industrialists to head UK foresight panels

London. Britain's Office of Science and Technology (OST) has moved to strengthen its links with the private sector by appointing five industrial scientists to head specialist 'technology foresight' panels, and a theoretical physicist turned management consultant to the post of part-time chairman of the Economic and Social Research Council (ESRC).

The industrial scientists will head five of fifteen panels whose details were released on Monday by William Waldegrave, the cabinet minister responsible for science. Each will cover a specific sector of social activity, ranging from transport, through leisure and education to defence and aerospace.

According to William Stewart, the gov-

ernment's chief scientific adviser and one of the architects of its foresight initiative, the panels will prepare reports that will then be used to "inform" government decisions about the allocation of research resources.

Stewart says that the OST has decided to go for an across-the-board approach, rather than starting more modestly as some had suggested with a few experimental foresight exercises, because "we felt it much better to commit ourselves to a major involvement."

The launching of the technology foresight panels coincides with the news that Bruce Smith, once an adviser to NASA on the Apollo space programme, is to be the new chairman of the ESRC. □

Japan trips up over high-definition television

Tokyo. A top official of Japan's Ministry of Posts and Telecommunications has caused uproar by suggesting that Japan should follow the United States and Europe and switch to the development of digital high-definition television (HDTV), rather than the analogue version developed in Japan.

There has been little surprise at the proposal to move away from the analogue system, even though the Japan Broadcasting Corporation (NHK) and Japanese industry have invested billions of dollars in developing it over the past 30 years. But the way in which the suggestion was made — which has led to any move being postponed for a year — has thrown some revealing light on the limitations of Japan's 'consensus' style decision-making.

It is now widely recognized in Japanese industry that digital HDTV transmission systems developed primarily in the United States offer many advantages over NHK's analogue system.

These advantages will increase following the widespread efforts around the world to develop multimedia interactive television, combining conventional television and computer technology. Europe abandoned its analogue system and switched to the digital approach more than a year ago.

But when Akimasa Egawa, director-general of the telecommunications ministry's broadcasting administration bureau, revealed last week that his ministry planned to follow this worldwide trend, he received criticism from all sides.

Last week (22 February) the *Nikkei Shimbun*, Japan's leading financial daily newspaper, reported that Egawa had told a meeting of a section of the Shinseito party, one of the governing coalition parties, that "the world trend is towards digital systems and the analogue approach has failed". He also hinted that Japan's test broadcasts of analogue HDTV might be halted.

At a hurriedly called press conference, Egawa toned down his comments. But he confirmed that the ministry hopes to revise its policy on HDTV by the summer, and added that continuing to promote NHK's analogue system without acknowledging the worldwide shift to digital systems amounted to "cheating the public".

NHK immediately issued a press release describing Egawa's comments as "very regrettable". This was joined by a chorus of protests from both industry and the Ministry of International Trade and Industry. Tadairo Sekimoto, chairman of the Electronic Industries Association of Japan, led the attack, and demanded the immediate withdrawal of Egawa's remarks.

(Ironically, Sekimoto is also president of NEC Corporation, whose researchers are furiously working on the development of digital systems, and his company probably

stands in one of the best positions to take advantage of a digital standard.)

Egawa's biggest crime appears to be his failure to win the consensus of industry and NHK before making his remarks, even though his views are in tune with many in his ministry and in industry.

He called a second press conference the next day to assure his critics that the ministry would continue to support the analogue standard, at the same time as moving towards digital systems. But that was not enough, and the Minister of Posts and Telecommunications, Takenori Kanzaki, had to step in and announce that a decision on HDTV will be postponed for a year while an advisory committee discusses the issue.

Behind the uproar is Japan's massive investment in analogue HDTV. NHK began development work in 1964, and has invested close to ¥20 billion (US\$200 million). Many electronics companies have in-

vested similar amounts and some analysts place Japan's total investment at ¥1,000 billion, although a more realistic figure is probably about half that amount.

NHK began experimental HDTV broadcasts of eight hours a day in 1991 and this will increase to 10 hours next month. But only about 20,000 HDTV sets have been sold, mainly because of their very high price — about ¥1 million each — and the limited HDTV programming that NHK can offer. A full broadcasting schedule is due to start using a new satellite in 1997, and is planned to run for at least ten years.

Both NHK and Japan's electronics industry clearly hope to squeeze as much return as they can out of their investment. But if Japan tries to hang onto the analogue system while the rest of the world goes digital, it may well come under pressure from its competitors to open up its market to their digital systems. **David Swinbanks**

Protests greet plutonium promotion

Tokyo. Attempts by the Japanese government to promote the use of plutonium in its nuclear power industry appear to have backfired. A promotional video made by the government-backed Power Reactor and Nuclear Fuel Corporation (PNC) to win public acceptance of plutonium has produced a storm of protest, including a request for its withdrawal from Hazel O'Leary, the US Secretary of Energy.

The video claims that it is safe for people to drink water contaminated with plutonium, and that there is no conclusive evidence that plutonium can cause cancer. But rather than reducing public opposition to the country's plutonium programme, the video seems likely to increase demands for the programme to be abandoned.

More than a hundred copies of the ten-minute video were distributed last year by PNC in Fukui Prefecture, the location of Japan's prototype fast breeder reactor Monju, which is due to attain criticality in a few weeks. It features a cartoon character, Pluto-kun, who tries to dispel the public's fears and "misunderstandings" about plutonium.

The most controversial scene in the film shows a gang of bandits dumping a large can of plutonium into a reservoir. Pluto-kun reassures viewers that plutonium is hard to dissolve, and that most will sink to the bottom. He says that even if someone drinks water from the reservoir containing plutonium, most of it will pass through the stomach and intestine, and then be excreted. And Pluto-kun is shown shaking the hand of a young boy gulping down a large glass of plutonium-contaminated water (see photo).

In another scene, viewers are warned that

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

"Drink up: it won't do you any harm"

it is dangerous to inhale plutonium or let it enter the bloodstream because it lodges in the body for years. But Pluto-kun adds that "unlike radium, there is no proven case of plutonium in the body causing cancer".

Early in February, O'Leary — who is leading a campaign that has highlighted the US government's involvement in radiation experiments on humans in the 1960s — wrote to PNC urging the company to withdraw the video.

Satsuki Eda, the head of the Science and Technology Agency (to which PNC is affiliated), acknowledged last week that there are some "inappropriate" sequences in the video. "Plutonium must never be leaked into the environment and must never be drunk," he said. But Eda has declined to order PNC to withdraw the video, claiming that the "inappropriate" parts "do not detract from the video as a whole".

PNC officials say that, although they are prepared to listen to comments, they have no intention of withdrawing the video. They argue that it is intended to use non-academic terminology to show the public that plutonium is not as frightening as some critics maintain. **David Swinbanks**

Censorship in geology?

SIR — *Nature's* recent review of Science in India drew attention once more to a Himalayan geological scandal: V. J. Gupta, professor of palaeontology in the department of geology at the Panjab University of Chandigarh, has been accused of scientific fraud carried out over a period of three decades (*Nature* 366, 616; 1993). The issues were first brought to the attention of a wide audience in 1989 by John Talent of Macquarie University, Australia (*Nature* 338, 613; 1989). These claims of scientific misconduct have not yet been satisfactorily resolved.

Just before *Nature's* recent review of Indian science, I had submitted to Elsevier's journal *Tectonophysics* a solicited book review of *The Phanerozoic Geology of the World; The Palaeozoic, A* (edited by M. Moullade and A. E. M. Nairn, Elsevier, Amsterdam, 1991). This book contains a lengthy chapter on the Phanerozoic of India, by V. J. Gupta and M. E. Brookfield. It was because of my comments about Gupta that Elsevier decided, in November 1993, against publishing the book review. In their words: "we honestly feel that the section about the Gupta chapter will bring us in the realms of legal implications". My "sensitive" section reads as follows:

In the final analysis of this book, however, the chapter on India requires the sharpest critical focus. India's Palaeozoic rocks are found in three distinct tectonic settings (viz. the High and lesser Himalayas, and cratonic India). The great stratigraphic contrast between the adjacent High Himalaya (formerly part of the northern margin of Gondwana) and lesser Himalaya, has long puzzled geologists, we are told. No real further insight is provided. Rather, what follows is a dour account of fossils and rock types. Most of the focus (~65% of the chapter) is on the High Himalayan sequences, whilst the classic Gondwana formations of the Indian craton are severely underthrust. The reason for this imbalance is because the major Palaeozoic controversies of India are found in the Himalaya; and the first author of this chapter is deeply embroiled in these controversies. Most of these controversies centre on biostratigraphy; Gupta and Brookfield claim that major errors have been made in the past: "These [other] workers claim to have collected all these fossils from a single stratigraphic horizon which, from the list, seem to be unbelievable and unacceptable. A thorough scrutiny of the genera and species recorded by these [other] authors indicates that the fossil identifications seem to have been made without taking any expert opinion on the subject" (p. 95).

The reader is referred to a great list of Gupta's own studies (all coauthored) for more reliable data. Yet this chapter's Gupta is the same Gupta who stands accused of corrupting the palaeontological literature on the Himalayas over the

past 30 years or so. The charges laid against Gupta (which include "salting" Himalayan rocks with foreign specimens, "recycling" fossils through different locations, and publishing misleading information about the location of fossil sites) were first made in 1988, and have since been openly debated.

Before submitting my review to Elsevier, I made certain that one of the editors (Nairn) had indeed been made aware of the Gupta affair several years before the book was to appear in print. Clearly the editorial office of Elsevier also knew well in advance of the publication date that Gupta was "on trial". Yet neither the editors (representing the scientific community) nor the publishers (serving the scientific community) were apparently willing to confront this thorny issue of scientific fraud.

From recent discussions at the headquarters of the Geological Society of India in Bangalore, I know that Indian geoscientists are concerned and embarrassed by the "irreparable damage to Indian science" that this saga may cause. B. P. Radhakrishna, president of the society, has also expressed his feeling that the "general silence [of the geoscience community at large] amounting to indifference is disconcerting" (*J. Geol. Soc. India* 35, 555–558; 1990 & 34, 561–563; 1989). Publication of this chapter suggests that he is right to feel let down by Western colleagues.

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Balanced board

SIR — You recently reported that the members of the board of the Danish National Research Foundation are no longer active scientists (*Nature* 366, 194; 1993). Fortunately this alleged fact is far from reality. Of the nine board members, six are indeed active scientists. In addition, two members, although not personally active in the laboratory, are responsible for extensive research activities. The balance on the present board between researchers and research administrators reflects the intentions of the Danish parliament when it created the foundation.

The board of the foundation looks forward with confidence to the evaluation of its activities scheduled for 1997.

Peder Olesen Larsen

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Shifting sands

SIR — F. J. Leavitt¹ asks four questions about sand movement. Only one, on the source of the sand in northern Sinai and Israel, can be answered convincingly.

The coastal sands in the northern Sinai and the Israeli coastal plain were shown in 1950 by Rim² (and subsequently confirmed by others), to derive from the submerged Nile delta, whence it is carried by longshore currents to the Mediterranean coast. The sand reaching the Sinai and Israeli coasts is eventually blown onto the beaches and inland. Most of it becomes arranged into transverse dunes and dune ridges, which have become a prominent feature of the coastal plain. Further inland, it changes into longitudinal (seif) dunes due to the strongly seasonal bidirectional winds^{3,4}. Seifs advance slowly by elongation at the dune front, without covering the interdune areas, and may even contain saline sebkhas when the water table is high. Palms and other vegetation in the interdune area thus remain uncovered because of the way in which the seif dunes advance. Because the bidirectional winds are not of equal strength, lateral movement of the sand body is very slow, leaving the interdune area uncovered for a long time.

Bushes and other plants in the interdune area have no special effect on deflecting the wind. If a structure is rather open they reduce the wind speed, and any sand carried will then be deposited in the wind shadow, on the lee side of the plants. When the obstacle is dense or compact, like a wall or plastic screen, the sand will accumulate in front of the structure and eventually cover it. On the basis of this simple principle, various devices have been designed and constructed to protect roads, railways, buildings and so on⁵. The best protection, of course, is to prevent sand movement at source, but this may be difficult or uneconomic, or, in the case of the Mediterranean coast, practically impossible.

The final question on what kind of ecological changes the control of wind-blown sand may bring about is very broad. Obviously man's interference or control of sand movement is not always beneficial or, rather, the results may be beneficial at one spot but detrimental elsewhere. In general, each system and project must be dealt with on its own merit.

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1. *Nature* 366, 198 (1993).
2. *Israel Exploration J.*, 1, 20–33 (1951).
3. Tsoar, H., *Z. Geomorph. Suppl. Bd.* 20, 41–61 (1974).
4. *Sedimentology* 30, 567–578 (1983).
5. *Techniques for Desert Reclamation* (ed. Goudie, A. S.), (Wiley, New York, 1990).

Shake-up for German universities

Hans Joachim Meyer

The right to a university education in any subject is enshrined in German law. But the system has become hopelessly cumbersome and is ripe for radical reform.

GERMAN higher education is characterized by two fundamental contradictions. First, German universities are institutions of mass education — the number of students has increased from 390,900 in 1960 to 1,823,200 in 1992. The University of Heidelberg alone has 29,400 students, almost exactly the same number as the whole of Germany in 1886. The biggest German university, Munich, has 63,500 students. But the principles of university admission and education are more or less still those of Wilhelm von Humboldt. He considered the *gymnasium* (the German high school) and the university as parts of one system, in which the former has the function of preparing for the latter. Accordingly, since that time, the leaving certificate of the *gymnasium* (the *Abitur*) guarantees the right to study any subject at any university. Moreover, the *Abitur* is thought to indicate the intellectual "maturity" necessary for students to organize their studies themselves. In today's complex and overcrowded universities, these two principles have become unworkable.

Second, although the rules of admission are identical for all German universities and although there is a central student distribution agency for degree courses which, because of the large number of students, have been declared "closed" (*numerus clausus*), there is no national agreement about the subjects required for the *Abitur* or on examination standards. In Germany, education, culture and science are the constitutional domains of the *Länder* (regions). The Federal University Coordinating Act (1976) states that the *Abitur* guarantees the right to university admission, but the examinations constituting the *Abitur* are regulated by each *Land* separately. Agreements between the *Länder* on matters of education, culture or science must be unanimous.

Difficulties

This explains why national reform of higher education is so difficult to achieve. In fact, although the recent reform proposals formulated by the Science Council and by the University Rectors' Conference have been welcomed both by the federal government and the governments of the *Länder*, no joint political action has been undertaken to put them into law. On the contrary, the education summit held last December was finally reduced to an ill-prepared item on the agenda of an

ordinary meeting of the federal chancellor with the prime ministers of the *Länder* — a painful event that brought no tangible results.

At the same time, however, some of the *Länder* have begun to reform their own university laws. The Saxony parliament was the first to pass a new university act embodying all the reform proposals — at least as far as the federal law permits.

In the course of German history, universities and university reform have often been the focus of debate in periods of national crisis, as was the case when von Humboldt defined the idea of the modern university. It occurred again during the revolution of 1848–49, which tried in vain to establish national unity on liberal and democratic foundations; and again at the time of the hapless Weimar Republic when, although bold projects of educational reform were designed, the conservative majority of professors and students despised democracy and thus paved the way for Hitler; and finally, in the late 1960s and early 1970s, when the students' revolt shook professorial power and initiated a far-reaching process of liberalization in West German society.

Observers of the German university scene may be tempted to consider the present debate about educational reform as part of the difficult internal unification process. But since the initial euphoria gave way to disillusion and sobriety, the challenges posed by unification have intensified a debate that had started in West Germany before 1989.

What East German universities can now contribute is a greater readiness to accept the practical relevance of teaching and research, and a resolve by faculty and students to make the best of difficult conditions. Although in the past such attitudes had been deliberately promoted as being in the interests of socialism, this political and ideological tinge has long faded away, if it was ever taken seriously. Unification helped to invigorate teaching and research in East German universities, the unity of which had been weakened by the concentration of research in the academy institutes, although — contrary to what is sometimes maintained — it had never been given up.

What can be learned from East German universities is that teaching and learning must be regarded not as the responsibility of the individual professor or student but rather as a common responsibility of both,

and of the professoriate as a whole. East Germany's role has been that of a catalyst of the structural crisis that had already developed in West Germany before the breathtaking events of 1989–90. The question is whether the desire for self-fulfilment can still be the justification for constantly expanding the rights of the individual, particularly in view of economic considerations. Or can the present crisis be overcome only by re-examining individual rights and privileges in the light of the common interest, and by reassessing structures and institutions in the light of their effectiveness for the needs of the individual and of society?

Distinctions

The main topic of the university reform debate is the length and structure of degree courses. In the eyes of the public, this is linked with the appallingly high number of drop-outs and long-term students, which suggests that the universities lack both energy and authority, and that they waste taxpayers' money. One measure proposed to tackle this problem is based on the distinction between 'pure' and 'applied' teaching and research, and suggests that the latter should be expanded more rapidly because it is more quickly effective for professional education and regional development. Another is to delimit the length of the degree course depending on the field of study or the type of academic institution, and to distinguish between professionally orientated first-degree courses and research-orientated (post)graduate studies.

Although it has repeatedly been emphasized that both stages of university studies should educate through academic work (*bildung durch wissenschaft*) and that the main difference should be the degree of structure in teaching, this proposal has provoked the wrath of those students and professors who prefer to see the university as a universe of discourse for a (possibly lifelong) unfolding of their personalities, or who simply find it difficult to agree on or to accept a clearly structured curriculum. Nobody will be surprised to learn that these voices are most often heard in the humanities and social sciences (a field I know well from personal experience).

The truth is that in an 'open' university the distinction between structured professional studies and less formally organized research studies may provide the only

chance for the university to prepare a student to contribute to society or to the academic community. But these aims can hardly be achieved without infringing upon long-cherished rights by, for example, introducing well-defined curricula (instead of collections of examination requirements); applying legal or financial sanctions against students who do not take their examinations in time; restricting the freedom of students to change their degree courses; enhancing the individual and collective responsibility of teachers for the success of their students; and linking support for research to the commitment of the professors to academic teaching.

A particularly controversial question is whether, in addition to the professors, there should be a special category of lecturers with a larger share in teaching. East German universities demonstrate the importance of experienced middle-rank academics in obtaining good teaching results. These institutions regret that university budgets now follow the West German pattern and have drastically reduced this group. Many in West Germany remember the fierce struggles in the late 1960s to define the teaching duties of middle-rank academics, and fear a repeat of disputes about pay and status.

Unfortunately, the demands for reform are intertwined with the equally justified demand for larger university budgets. Since the late 1970s, West German universities have been forced (and seduced by temporarily limited special funds) to carry an overload of teaching, because the governments of the *Länder* have clung to the unrealistic hope that the "students' mountain" would disappear. East German universities show signs of long neglect and mismanagement, and are in sore need of modern equipment. Thus the temptation is irresistible to give priority either to increasing the money, as demanded by students and professors, or to introducing reforms, as urged by local and national governments.

The governments themselves are divided as to which bears the larger share of political responsibility—the governments of the *Länder*, which would have to introduce most of the legal reforms and to face angry protests, or the federal government, whose constitutional position in higher education is very weak but which has to provide half the money for new buildings and equipment. On top of that, the governments of the *Länder* are widely different in their educational policies, and disagree on which reforms should come first or whether they should come at all.

An almost equally prominent and controversial topic is the demand for the reform of university organization and governance. None of the teaching reforms can be brought about without strong leadership within the universities themselves.

Effective academic leadership, however, tends to be regarded with distrust by both professors and students, who want to preserve their traditional privileges or to guarantee the rights of minorities and individuals. The reform movement of the late 1960s and early 1970s demanded democratic procedures requiring the consent of all university groups involved. This concept owed too much to class-struggle ideology and not enough to teaching and research.

The attempt at radical democracy in universities has been thwarted by a ruling in 1973 of the federal constitutional court in favour of the profession by the Federal University Coordinating Act and by the corresponding University Acts of the *Länder*, which have often increased the influence of ministerial bureaucracy. As a result of these conflicting developments, the decision-making processes in universities have often become long, ineffective and, for ordinary university members, a disincentive to participate in university affairs. It would, nevertheless, be a grave mistake to return to the old German *ordinarienuniversität* which was often not much more than a confederation of independent professors with a *rektor* and with deans who had to be elected each year.

Coordination

Instead, an effective system of university government is needed, which combines democratic participation with the ability to act swiftly and energetically. Electoral procedures and the structure of elected bodies must be designed to take into account the pragmatic needs of staff and students. It is therefore vital to coordinate decision-making at the faculty or departmental level as well as at the university level. The *rektor*, the *prorektoren* and the deans should be elected for a term of not less than 3 years, while the *kanzler* (head of university administration), who is responsible for the budget, must hold office for a much longer period.

The faculties, provided they combine a number of related and adjacent disciplines, must become the pillars of the university structure. The *dekane* (deans) should be the central figures of effective academic leadership in the university. For this they need the support of a *prodekan*, some *studiendekane* (students' deans) and an administrative officer, and must have disciplinary as well as financial rights (at present an unheard-of privilege). Last but not least, a clear distinction is necessary between bodies taking decisions on academic matters and those deciding on finance, to minimize the danger that meagre means are distributed by a grand coalition of diverse special interests. Thus, academic matters could be dealt with by the university senate, whereas all important financial decisions could be taken by a university board, which should

be advised by independent personalities.

The most important reform, without which the others would not make much sense, must concern the principle of university admission by right to all high-school graduates. The consequences of this principle are disastrous, but it is nonetheless passionately defended by ideologues and lobbyists of very different colours, either as an instrument of achieving an egalitarian society or as a shield for protecting the prestigious role of the traditional German *gymnasium*.

There are two ways out of this dilemma. Either the German *Länder* must agree on a range of compulsory subjects (for example, German, mathematics, a foreign language, a science subject and a humanities subject) and on mutually accepted standards for the *Abitur*. Or the Federal University Coordinating Act must be changed so that each *Land* can specify school subjects that are required in the *Abitur* certificate for admission to particular degree courses. In view of the German constitutional principle of cultural federalism, the second course seems to be more realistic. It may also have the advantage of triggering competition among universities to attract the best students. It may also induce competition among the German *Länder* for centres of excellence in university teaching, which, in turn, may make taxpayers more sympathetic to universities' requests for more money.

In July 1993, the Saxony parliament passed a Higher Education Act that included practically all these reform proposals for structuring degree courses effectively and for strengthening university teaching. The new law restored academic autonomy on a democratic basis and, at the same time, established a new model of governance intended to ensure effective academic leadership. The new law even has a clause to allow universities and colleges to influence young people at the *gymnasium* in their choice of school subjects for the *Abitur* in the light of the degree courses that interest them. However, this can go only as far as is allowed by the federal law and the state treaty on university admission between all German *Länder*. What is really needed is a national consensus on university policy. Because university policy is, and should remain, the constitutional domain of the *Länder*, which invariably follow different educational concepts, the purpose of the consensus cannot be uniformity, but diversity. Diversity makes sense only if it means competition. What we need in academic teaching and research, as well as in educational policy, is more competition. □

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A modest proposal for Irish renewal

Communal divisions in Northern Ireland, responsible for twenty-five years of violence, might be partly bridged by an imaginative scheme for a new campus in Belfast. But why not go further?

Belfast. What is the obligation of universities to the social environment in which they find themselves? The most common answer is that the education of the young is a sufficient justification. Where would a society be if it could not easily acquire the skills to keep industry and the social infrastructure alive and capable of innovation? On that view, it is simply a by-product of higher education that the same young people may have the intellectual breadth to change from within the societies to which they belong. The creation of new knowledge is something else again, a tribute to international scholarship. That, in normal circumstances, is the case for the modern university.

But what if the circumstances are abnormal? Last weekend, Sinn Féin, the Republican Party, was meeting in a muddy tent on the outskirts of Dublin to decide (among other things) what response to make to a declaration on the future of Ireland by the British and Irish governments. In the event, Sinn Féin said nothing that will carry weight in London or Dublin. The violence will go on.

Not that Belfast seems a violent place. The murder rate is less in the whole of Northern Ireland than in, say Washington, DC, and by a substantial factor. A quarter of a century of the present troubles notwithstanding, most people seem remarkably cheerful. There are (British) Army patrols on the streets and policemen at the entrances to most public buildings, but the city itself looks exceedingly well; bombed buildings are quickly rebuilt, while the new public housing on the sectarian frontier is a credit to the British government (and the British taxpayer, who has paid for it). And Belfast, with its sea lochs and suburban mountain-scapes, is as spectacular a piece of topography as ever.

But it is tiny. The provincial capital, with 300,000 people, houses a fifth of the population of Northern Ireland, which itself accounts for 30 per cent of the 5 million or so in the whole of Ireland. If the nationalists were to win their united Ireland, there would be fewer of them than there are Swiss. What arrangements would they make for higher education? And how should the existing universities order their affairs to accommodate the uncertainties ahead?

Ireland's universities are the island's history in bricks and mortar. Trinity College, Dublin, was an Anglican foundation in 1595, from which Presbyterians as well as Roman Catholics were excluded. Queen's University Belfast is the surviving eponymous

relic of the Queen's University of Ireland, created in the mid-nineteenth century (in partnership with the still-extant universities at Cork and Galway in the republic). The National University of Ireland began life in Dublin (in 1851) as the Catholic University of Ireland (now University College Dublin), but has now absorbed Cork and Galway. The most recent creation is the University of Ulster, formed in 1984 by the merger of the previous New University of Ulster (formed in the mid-1960s at Coleraine and Londonderry) and the Ulster Polytechnic (sited in Belfast).

At least by British standards, most of these institutions are substantial. Queen's College Belfast and University College Dublin have about 10,000 students of all kinds, while the University of Ulster has quickly grown to the same number. Ireland in total has about 50,000 students at any time, a third of them in Northern Ireland. Queen's Belfast has by far the best record on research, earning some £8 million from research grants two years ago.

Most of these institutions are partly imprisoned by their origins. Early in the present troubles, Queen's Belfast, under vice-chancellors such as Sir Arthur Vick, took it as its social role to be an island of calm in a sea of turmoil; research aimed at understanding what was happening on the streets was discouraged. Much has now changed, but Queen's is still somewhat timorous, perhaps overconscious of its Protestant traditions. In different ways, most other Irish universities exude the same defensiveness.

The exception is the University of Ulster, which has quickly won a reputation for being go-ahead, some say for bravado. It boasts of the best facilities for computer networking in the United Kingdom as well as of a joint programme with the United Nations University on "conflict resolution and ethnicity". More than 1,000 of its students are from south of the border.

Last September, the university startled even its own members with an audacious plan: to build a new campus for more than 4,000 students on a derelict industrial site spanning what is called the peace line at Springfield in West Belfast. Over many years, the British government has been unsuccessful in persuading industry to build factories there. The scheme, which is chiefly meant to meet the growing demand for higher education, is also political. One of the questions for the feasibility study now under way is whether a campus so sited would attract students from both the union-

ist and the nationalist communities.

The scheme has been welcomed as a "visionary initiative" by Sir Patrick Mayhew, the British government's Secretary of State for Northern Ireland. Even the politicians have been cheerful about the project, the nationalists perhaps the more so. The government also seems to be taking the project seriously. Academics elsewhere are less sanguine, fearing that the cost may diminish their budgets. But these are early days. There are hopes that in the end, some part of the £100 million or so the development might cost could come from Brussels, not London.

What are the chances that the project will help bridge Northern Ireland's conflict? In the end, local politicians and not academics will decide that. But it is not irrelevant that the communal conflict has not obliterated the sense that Ireland is one island. Both parts of it compete internationally as one in horse-riding and rugby football (in which respect Ulstermen last weekend were still savouring "our victory" over England). Dublin still has a "Royal Irish Academy" and the flux of students between the two is substantial. Students trickling South are supported financially by their local authorities, students moving North have their fees paid by the British government. Cross-border research projects are increasingly common, usually on the initiative of individuals.

The University of Ulster's Springfield campus, if it comes off, would dramatically bolster that sense that the communal conflict is at some level an irrelevance. But why not go even one step further? Ireland's 5 million people now have two university systems, one run from Belfast and one from Dublin. Why not put them all together, under a single funding council to which both governments would contribute? The benefits would be both to prove that higher education is above politics and to demonstrate that its institutions can, by their existence, help solve political problems. No institution would be required to abandon its traditions. All would profit from being players in a bigger pool.

Already, it seems, the heads of all the Irish universities have held two meetings, piquantly this year at the Catholic University of Louvain in Belgium. It would make more sense if they were meeting every month or so, in Ireland, to talk about their common problems. The British and Irish governments are looking for projects on which to work. Here is one.

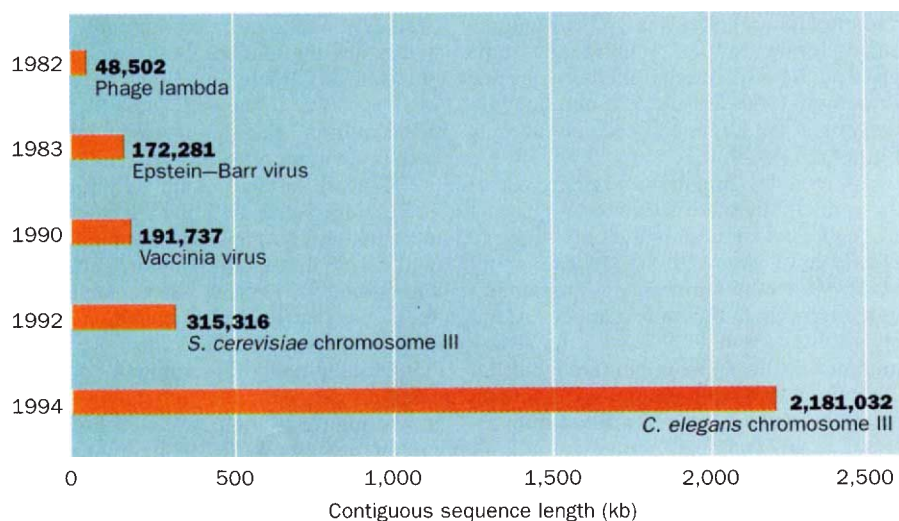
John Maddox

Back to bases in biology

Stephen Oliver

ON page 32 of this issue¹, a joint team from the Genome Sequencing Center (St Louis, USA) and the newly founded Sanger Centre (Hinxton Hall, Cambridge, UK) report a contiguous sequence of over two megabases from chromosome III of the nematode worm, *Caenorhabditis elegans*. This is the longest contiguous DNA sequence yet determined, and it prompts rumination on how far we have come in the sequencing enterprise, and on how far — and where — we have to go.

are all, or part, of eukaryotic chromosomes. The data libraries contain a number of complete sequences of organelle genomes, but the longest contiguous sequence yet entered for a bacterial genome is 176 kb of the *Escherichia coli* chromosome. This reflects the current preoccupation with eukaryotic molecular biology, but the completion of the entire sequence of *E. coli* will provide molecular geneticists with a resource of enormous value.



Successive longest sequences of contiguous DNA entered in the public data libraries, starting with that of bacteriophage λ (ref. 2). The others are the complete sequences of the Epstein-Barr virus³, vaccinia virus⁴ and *S. cerevisiae* chromosome III (ref. 5), and the partial sequence of *C. elegans* chromosome III (ref. 1).

The figure shows the record of the longest sequences to date. The first thing to note is that the two most recent examples were determined by international teams, and that no doubt will be the pattern in the future. The second is the success of Fred Sanger's dideoxy sequencing method, and of the research school that he established at the Medical Research Council laboratories at Cambridge. All five longest sequences were determined by Sanger's method and three by graduates of the Sanger school. So there have been no dramatic breakthroughs in sequencing methods since 1977. What has allowed the move from kilobase (kb) to megabase (Mb) sequencing is the automation of the process. Only 7 per cent of the previous longest sequence — the 315 kb of *Saccharomyces cerevisiae* (yeast) chromosome III published in 1992 — was determined using automated methods, whereas the entire 2.1 Mb of the nematode's chromosome III has been established by machine sequencing.

The first three longest sequences were all viral genomes, whereas the last two

are all, or part, of eukaryotic chromosomes. It might have been expected that human genome sequences would feature very heavily in this league table. In fact, the longest sequence of human DNA lodged in the data libraries is just 73 kb of the β -globin region. This is the result of the emphasis on complementary DNA, rather than genomic, sequencing in the Human Genome Project. This is a sensible strategy when dealing with a genome of 3,000 Mb which is rich in repetitive sequences. But there are important data which can only be gained from sequencing chromosomes, for instance data relating to the higher order organization of both the genome and its genes, the rules governing transposition and recombination, and the principles of genome evolution. For instance, Wilson *et al.*¹ report on part of a homeobox gene complex which extends over 270 kb and which is now revealed to be related to the *Antennapedia* complex of the fruit fly *Drosophila*. They also provide examples of gene duplication and exon shuffling which may indicate how genes with novel functions are able to evolve.

The nearly 50-fold increase in the length

of the longest known DNA sequence between 1982 and 1994 bears testimony to the central role which genome sequencing holds in the biology of the late twentieth century. However, although sequencing is a technologically sophisticated activity, in conceptual terms it represents a return to biology's eighteenth- and nineteenth-century roots. Then, biologists roamed the world collecting organisms; today they stay at home in their laboratories, collecting genes. When our Victorian forebears had sampled the biological diversity of this planet, they realized that they did not understand it. Their first, and quite sensible, reaction was to classify what they had found. From this exercise, a new problem — that of the origin of species — emerged and was solved by Darwin's theory of evolution.

Today's collectors are discovering new genes at a rate which far exceeds that previously achieved by either classical or molecular genetics. Model organisms are particularly rich sources of new genetic information — a gene is discovered about every 2 kb in the genome of *S. cerevisiae* and about every 6 kb in that of *C. elegans*. Just 3 per cent of the nematode genome has been sequenced so far; this means that there are about 16,000 genes still to discover. In the case of *S. cerevisiae*, chromosomes III and XI (a total of nearly 1 Mb) have been completely sequenced by the European Union network. The sequences of chromosomes II (EU), VI (Japan), IX (UK), I (Canada), V and VIII (USA) are nearing completion. So, in a few months, 3.7 Mb of yeast chromosome sequences will be in hand. This represents roughly one-third of the total unique sequences in the yeast genome and should contain about 1,800 putative protein-encoding genes.

However, the startling (and challenging) thing is not the sheer number of genes which are being discovered, but that at least half of them are of completely unknown function. This is true not only of the information emerging from the yeast and nematode projects (the sequence reported in this issue defines 483 putative genes, at least two-thirds of which are of unknown function), but also of that from all of the genome projects, including those on the intensively studied bacterial genomes of *E. coli* and *Bacillus subtilis*.

The functions of these genes may be unknown, but they are probably important. Examples where genes with highly similar predicted protein products are found in the yeast, nematode and human expressed-sequence-tag data libraries are being found with increasing frequency. This presents modern biology with a

1. Wilson, R. *et al.* *Nature* **368**, 32–38 (1994).

2. Sanger, F. *et al.* *J. molec. Biol.* **162**, 729–773 (1982).

3. Baer, R. *et al.* *Nature* **310**, 207–211 (1984).

4. Goebel, S. *et al.* *Virology* **179**, 517–563 (1990).

5. Oliver, S. *et al.* *Nature* **357**, 38–46 (1992).

similar challenge to that faced by the Victorians: how to make sense of this plethora of information. By analogy, we will need to establish a taxonomy of gene function. This will require the integration of computational and experimental techniques in approaches which need to be as systematic and efficient as those of DNA

sequencing itself. But half of biology is still to play for, and the hope must be that general truths as compelling as Darwin's theory will be revealed. □

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CALCIUM CHANNELS

Taking the beta test

Bruce Bean

VOLTAGE-dependent calcium channels are composed of several subunits. But until recently most attention has centred on the largest (α_1) subunit, which comes in many varieties and contains both primary voltage sensors and the channel's selectivity filter. Now, two groups have made dramatic advances in understanding how α_1 interacts with a smaller (β) subunit, whose role has been less clear. At the structural level, Pragnell *et al.* (page 67 of this issue¹) have used an ingenious technique to identify a specific region of the α_1 -subunit that forms a binding site for the β -subunit. At the functional level, Neely *et al.*² have found that the β -subunit modulates the coupling of voltage sensors to channel opening.

Multiple types of Ca^{2+} channels are involved in a wide range of cellular functions, but the subunit structure is known for only a few, notably the principal skeletal muscle Ca^{2+} channel (which mediates excitation-contraction coupling and is blocked by dihydropyridine drugs such as nifedipine and nitrendipine). The dihydropyridine-sensitive skeletal muscle channel consists of four subunits³ — α_1 (relative molecular mass 175K), $\alpha_2\delta$ (160K), β (52K) and γ (32K).

The α_1 -subunit is composed of four homologous 'repeats', each with six predicted transmembrane regions including an S4 region that may form the voltage sensor of each repeat. In other tissues, molecular cloning has identified different α_1 -subunits homologous to the skeletal muscle α_1 -subunit, with six main classes being characterized so far⁴. The β -subunit is believed to be cytoplasmic, and four varieties are known⁵. The $\alpha_2\delta$ -subunit is predicted to include a single transmembrane region and occurs in brain as well as muscle. The γ -subunit seems to be unique to skeletal muscle. Of brain Ca^{2+} channels, detailed subunit structure is known only for the ω -conotoxin-sensitive (N-type) channel, which consists of an α_1 -subunit, a β -subunit, an $\alpha_2\delta$ -subunit and two smaller subunits not found in the skeletal muscle channel⁶.

At first glance, the goal of finding a specific region of the huge α_1 -subunit that binds to the β seems almost impossible. A

conventional mutagenesis approach might be endless, with few clues about where to start. Instead, Pragnell *et al.* took what might be called a 'smash and bind' strategy. They prepared random fragments of the skeletal muscle α_1 -subunit by digesting α_1 complementary DNA with DNAase, and formed an 'epitope library' of fusion proteins from some 20,000 small fragments. Gambling that single small fragments might be sufficient to form a binding site for the β -subunit, they screened the cloned fragments with radiolabelled β -subunit and identified seven positive clones. All of these pieces turned out to share a 45-amino-acid overlap that maps to the cytoplasmic loop forming the link between repeat I and repeat II of the α_1 -subunit.

In experiments on three other α_1 -subunits (encoded by different genes), all β -subunit-binding epitopes mapped to the same region. Comparison of the epitopes led to the identification of an 18-amino-acid binding motif. The similarity of this small motif in the different α_1 -subunits is especially striking because the sequences of the I-II linker region as a whole have little in common in the different channels. Mutations of four of the conserved residues of the motif reduced the ability of the β -subunit to bind to a neuronal (class A) α_1 -subunit, and also its ability to enhance the Ca^{2+} channel current expressed by this α_1 -subunit in *Xenopus* oocytes.

What are the functional consequences of β -subunit binding? Co-expressing β -subunits with α_1 -subunits alters the voltage-dependence, kinetics and magnitude of the Ca^{2+} channel current, with different effects depending on the particular β - and α_1 -subunits used (reviewed in ref. 4). The most detailed analysis has been carried out on cardiac subunits expressed in *Xenopus* oocytes. Relative to the current expressed by injection of the cardiac α_1 -subunit alone, co-expression of the cardiac β_2 -subunit results in an increase in peak current (of some four times), a speeding of activation kinetics and a shift in the voltage-dependence of channel activation in the hyperpolarizing direction^{7,8}. The simplest interpretation

of these effects would be an increase in the number of expressed channels, along with a change in voltage-dependent gating.

In this context, Neely *et al.*² have provided an illuminating surprise. As well as measuring the ionic currents through the Ca^{2+} channels, they also recorded gating currents (the small currents arising from intramembrane movement of the channels themselves as they change conformation in response to changes in membrane voltage). The gating current from co-expressed cardiac α_1 - and β -subunits was essentially unchanged in both magnitude and voltage-dependence from that produced by α_1 -subunits alone, even though ionic current was enhanced and shifted in voltage-dependence. From these results it seems that the presence of the β -subunit does not alter the number of expressed channels or the movement of the voltage sensors of the channels. Instead, the β -subunit somehow facilitates coupling between movement of the voltage sensors and opening of the channel pore. The ionic conductance for large depolarizations is enhanced some five times by co-expression of the β -subunit, so when the α_1 -subunit is on its own the probability of channel opening is apparently quite low (less than 0.2) even after complete movement of the voltage sensors. The 'coupling efficiency' between voltage sensors and pore opening is increased by the β -subunit.

There is now an interesting puzzle concerning dihydropyridine binding to the cardiac α_1 -subunit. When β -subunits were co-expressed in cultured mammalian cells, the number of high-affinity dihydropyridine-binding sites (thought to be on α_1 -subunits) dramatically increased⁷⁻⁹. Yet the gating current experiments of Neely *et al.* suggest that there is no change in the number of functional α_1 -subunits, and this is supported by other experiments showing no change in levels of expressed α_1 RNA or protein⁹. Binding of the β -subunit may somehow alter dihydropyridine binding to the α_1 -subunit, although β -subunit expression is clearly not required for high-affinity binding⁷⁻⁹. Neely and colleagues suggest that β -subunits might alter the equilibrium of α_1 -subunits between two different states, one sensitive and one relatively resistant to dihydropyridines. It should be possible to test this idea by examining the dihydropyri-

1. Pragnell, M. *et al.* *Nature* **368**, 67–70 (1994).
2. Neely, A. *et al.* *Science* **262**, 575–578 (1993).
3. Catterall, W. A. *Science* **242**, 50–61 (1988).
4. Snutch, T. P. & Reiner, P. B. *Curr. Opin. Neurobiol.* **2**, 247–253 (1992).
5. Castellano, A., Wei, X., Birnbaumer, L. & Perez-Reyes, E. *J. Biol. Chem.* **268**, 12359–12366 (1993).
6. Witcher, D. R. *et al.* *Science* **261**, 486–489 (1993).
7. Lacerda, A. E. *et al.* *Nature* **352**, 527–530 (1991).
8. Varadi, G., Lory, P., Schultz, D., Varadi, M. & Schwartz, A. *Nature* **352**, 159–162 (1991).
9. Nishimura, S. *et al.* *FEBS Lett.* **324**, 283–286 (1993).
10. Reuter, H. *J. Physiol., Lond.* **192**, 479–492 (1967).
11. Klockner, U., Itagaki, K., Bodi, I. & Schwartz, A. *Pflügers Arch.* **420**, 413–415 (1992).

dine sensitivity of gating current in the presence and absence of β -subunits.

Because β -subunit binding to the α_1 -subunit modulates the gating of the Ca^{2+} channel, it will be interesting to see whether the interaction between the subunits can be altered by phosphorylation or other influences such as G proteins. Cardiac Ca^{2+} current is increased by β -adrenergic stimulation¹⁰, and the β -subunit may be needed for this

modulation¹¹. If so, the present line of research may lead to a molecular understanding of the original example of a voltage-dependent ion channel whose behaviour is modulated by neurotransmitters. □

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POLYMER CHEMISTRY

Coming to an unsticky end

Robert F. Brady Jr

THE non-stick surface — a surface that resists or even repels clinging matter and never needs cleaning — may be the chemical equivalent of the perpetual motion machine: earnestly desired, but unattainable. Creating such a surface requires careful control of the composition,

faces. The coatings must, of course, be safe to manufacture and use; in our drive for cleanliness we do not wish to taint either our bodies or our environment.

In a new approach to coatings with low-energy surfaces, Schmidt and co-workers polymerize perfluorooctyl acrylate or perfluorooctyl methacrylate esters with monomers containing ionizable functional groups to create a fluorinated polymeric surfactant. In solution this polymer spontaneously organizes itself to present the fluorinated groups to the air–water interface and the ionic groups to the substrate (see figure). At this temporary stage the coating is not wetted by liquid hydrocarbons, but redissolves in polar liquids because ionic groups remain unreacted.

The authors prepare robust coatings by reacting this polymer with a water-compatible crosslinking

polymer; they use a polymer with pendant oxazoline rings for this purpose. A mixture of the two polymers is stable in a water–glycol solution, but forms a sturdy crosslinked film when applied to a surface and cured at 130 °C for an hour. Synthetic adhesives do not stick to the film, and it is not wetted or attacked by common solvents. A second coat of the same material adheres well, however, because fluorinated groups collect at the interface where they align with the fluorinated groups in the first coating.

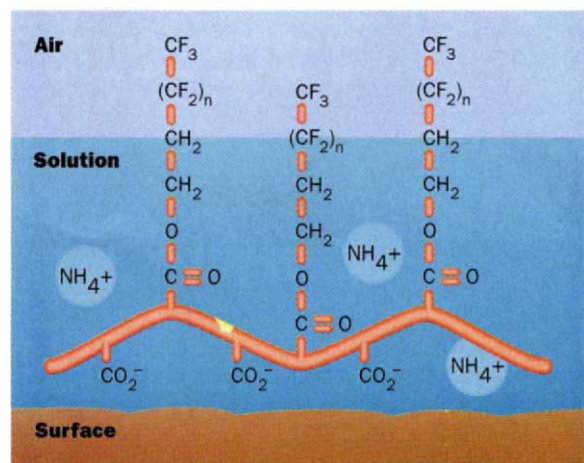
The concept that a low-energy surface is best achieved by closely packing and orienting perfluoroalkyl (R_F) groups is evolutionary, rather than revolutionary. Surface energy is known to be related to the functional groups at the polymer surface⁴; among polymers in common use, the hydrocarbon-based poly(ethylene) and poly(propylene) have the lowest

surface energies. But when fluorine is substituted for hydrogen, the surface energy is reduced further. Electrons are held closely and tightly about the fluorine nucleus and cannot be shared or easily polarized, whereas the electron density around the hydrogen nucleus is mostly in the covalent bond, leaving the nucleus unshielded and able to induce weak bonds. Thus substitution of fluorine causes the surface energy to decrease in the order $-\text{CH}_2- > -\text{CH}_3 > -\text{CF}_2- > -\text{CF}_3$. This is readily observed in the surface energies of polymers with surfaces composed of ordered single groups such as poly(ethylene) (33.7 mJ m⁻²), poly(dimethylsiloxane) (21.2), poly(tetrafluoroethylene) (PTFE) (18.6) and poly[di(3,3,3-trifluoropropyl)siloxane] (6.0).

Many once believed that PTFE, the most familiar of the non-stick polymers, would be the answer to their prayers, but its usefulness is limited. PTFE is not very easily processed because it is not dissolved, softened or even wetted by common solvents. It is effective in cookware and some other applications but it accumulates all types of marine fouling with astonishing speed because of its porosity — marine adhesives invade cavities in the surface and cure inside them, creating a secure mechanical interlock⁵. There is a need for a coatings technology that can deposit a non-porous PTFE-like surface wherever desired. This technology will have to comply with a host of contemporary environmental and human health regulations such as the reduction or elimination of air-polluting organic solvents in favour of water-based technologies. Schmidt and co-workers have made an impressive move in this direction.

Producing polymers with a large number of R_F groups is relatively easy, but causing these polymers to form ordered films with the R_F groups aligned and closely packed on the surface is far more difficult. The authors use ethylene glycol (or another polar liquid) to plasticize the film, keeping it pliant as crosslinking progresses and providing time for systematic orientation of the R_F groups. Polar liquids also improve surface smoothness, another important contributor to non-stick behaviour. Surfaces in air, water and hexadecane, studied using scanning force microscopy, show a peak-to-valley roughness of 0.4–1.3 nm which is independent of environment or crosslink density.

After the R_F groups form on the surface, they may not stay there, for liquids on the film may cause the R_F groups to migrate into the film. Schmidt and co-workers use contact angle studies with hexadecane and water to evaluate the orientation and mobility of the R_F groups and to obtain an estimate of the surface free energy. A series of coatings is studied



Coming unstuck — the favoured conformation of a fluorinated surfactant directs hydrophobic groups towards the air–water interface and ionic groups towards the substrate.

free energy, texture and electrical properties of the surface, and these parameters must be optimized for each application. Current research on non-stick coatings focuses on minimizing their surface free energy, for this is believed to be the dominant factor controlling anti-adhesive behaviour. On page 39 of this issue, Schmidt and co-workers¹ report an ingenious water-based polymerization technique to produce a hydrophobic coating with an unusually low surface energy, about 11–16 mJ m⁻². The hard, clear coating is not wetted by solvents and does not attach to adhesives.

Non-stick coatings are crucial for many applications, including peel-off backings for self-stick labels², prosthetic devices that do not accumulate biological debris, bearings and valves, non-toxic fouling-release coatings for ships' hulls³, stain-resistant fabrics and self-lubricating sur-

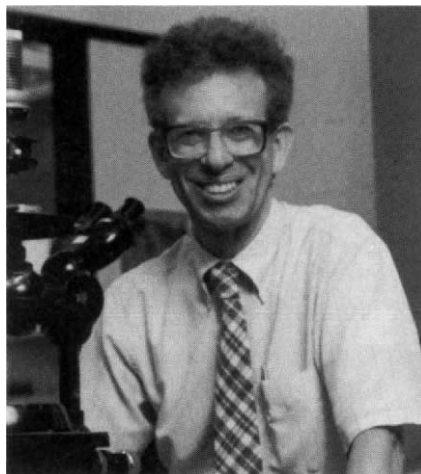
Howard M. Temin (1934–94)

ON 9 February, Howard Temin, recipient of the Nobel Prize in Physiology or Medicine in 1975, virologist and professor of oncology at the University of Wisconsin, died of metastatic adenocarcinoma of the lung.

Temin's many scientific contributions are crowned by his concept, developed in the mid-1960s, that RNA tumour viruses reproduce by a process involving an integrated DNA intermediate. He called this DNA intermediate the provirus, by analogy with the prophage of a bacterial virus; in retrospect, his idea of a provirus, based at first only on indirect laboratory results, was one of those rare moments in science when one man's insight drags a whole field into a new realm of thought and pushes aside bottlenecks in several other areas. But for several years he was constrained by disbelief and criticism, until the dramatic and independent discoveries in 1970 by Temin and Mizutani (*Nature* 226, 1211; 1970) and by David Baltimore (*Nature* 226, 1209; 1970) of the enzyme reverse transcriptase, a singular DNA polymerase that provides the catalytic activity for transcribing viral RNA into DNA. The enzyme of Temin and Mizutani was from a chicken retrovirus whereas Baltimore's was from a mouse leukaemia retrovirus, but it was soon learned that reverse transcriptase is carried by every infectious RNA tumour virus.

The way was now open to determine the replication cycle of these cancer-causing animal viruses and the strategies they use to transform cells, but this was only the start of the impact of reverse transcriptase on molecular biology. The unidirectional flow of genetic information, DNA to RNA to protein, had until

then been taken for granted. Further, reverse transcriptase became a key tool in biotechnology by virtue of its ability to synthesize DNA copies from any messenger RNA template. Finally, measuring the activity of the enzyme soon became a sensitive and specific way to assay for a retrovirus and was one of two crucial methods that led to the discovery of human retroviruses, the leukaemia



Howard Temin, Nobel prize winner in 1975.

viruses (HTLV-I and HTLV-II) and the human immunodeficiency virus responsible for AIDS.

Temin began his postdoctoral fellowship at Cal Tech with Harry Rubin and Renato Dulbecco in 1956 (Dulbecco and Baltimore shared the 1975 Nobel prize with Temin). He started studying the transformation of cells by the Rous (avian) sarcoma retrovirus when he realized that this *in vitro* phenomenon, then just described by scientists at Rutgers University, was an opening to under-

standing cellular alteration, an interest he maintained throughout his career. In 1960 he moved to Madison to join the cancer research centre at the McArdle Laboratory in the University of Wisconsin, where he remained a member of this renowned group. In recent years he turned his attention to mechanisms of generating variation in retroviruses, central to the problem of drug resistance and vaccine development for AIDS. He continued to be active as a teacher, researcher and advisor to the National Cancer Institute (as a member of the National Cancer Advisory Board) until only weeks before his untimely death.

Howard Temin's mind held a rare combination of creativity and penetrating, analytical insights, and his judgement was a rare combination of compassion and independent objectivity. In an era when biological laboratories have grown in size, needs and complexity, his team maintained a focus on individual creativity. Despite the modern pressures on families, he was a devoted family man — to his wife, Rayla Greenberg Temin (geneticist at the University of Wisconsin), and to his daughters, Sarah and Miriam. Finally, at a time when biomedical scientists in the United States are more and more involved in entrepreneurial adventures, Temin remained where he began, the pure academician. Once when I asked him why, his reply was a typical Teminism: "With all those experiments going on out there, there must be at least one control!"

Robert C. Gallo

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in which the ratio of fluorinated polymeric surfactant to crosslinking agent is varied between 0.3:1 and 3:1. As the films become more highly crosslinked, the concentration of R_F groups at the surface decreases, and these groups are less able to reorient themselves. The surface free energy lies between 11 and 16 mJ m⁻², indicating that the surface is indeed composed of a preponderance of R_F groups. X-ray data are consistent with layers of R_F groups about 8 nm thick.

The new synthetic scheme is versatile enough to allow production of surfaces with a range of energies. A series of coatings with tailored surface energies could be made by varying the monomer ratios in the reactants, perhaps using non-fluorinated monomers when higher surface energy is desired, and used to isolate and explore the influence of surface free energy, which is widely believed to be the primary determinant of ad-

hesion. Although synthetic adhesives show a reasonable (but not perfect) inverse relationship between bonding strength and surface energy, natural glycoproteins and polysaccharides show the least adhesion within a range of surface energy which is near, but does not include, the lowest values. Baier and Meyer⁶ recommend 20 to 30 mJ m⁻² as the optimum range for the free energy of a surface designed to shed biofouling and say that efforts to reduce the surface energy below this range are counterproductive.

Before we conclude that the ultimate non-stick coating is at hand, silicone and urethane chemists must have their innings. Compared with a carbon backbone, the O-Si-O backbone has a more open structure, with a flatter angle and a longer bond length; available surface-active groups on a silicone backbone adopt the lowest surface-energy configuration much more readily. Silicone coatings will need

improved mechanical strength for some applications. Elastomeric urethane coatings, however, combine backbone flexibility with good mechanical properties. My view is that the consummate non-stick coating will have a silicone backbone, but I'm not yet prepared to bet whether it will contain fluorine. □

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- Schmidt, D. L. *et al.* *Nature* **368**, 39–41 (1994).
- Owen, M. J. *J. Coatings Technol.* **53** (679), 49–53 (1981).
- Brady, R. F. Jr, Griffith, J. R., Love, K. S. & Field, D. E. *J. Coatings Technol.* **59** (755), 113–119 (1987).
- Zisman, W. A. in *Contact Angle, Wettability, and Adhesion*, *Adv. Chem. Ser.* **43**, 1–51 (Am. Chem. Soc., Washington DC, 1964).
- Saroyan, J. R., Lindner, E., Dooley, C. A. & Bleile, H. R. *Indust. Engng Chem., Prod. Res. Devel.* **9**, 123–128 (1970).
- Baier, R. E. & Meyer, A. E. *Biofouling* **6**, 165–180 (1992).

Connexins in disease

Michael V. L. Bennett

KNOCKOUTS can tell a lot about a gene's function. But they are perplexing if it turns out that loss of function of a seemingly important gene still results in a fairly normal phenotype. Gap-junction researchers are in just this situation now that two groups^{1,2} have shown that a relatively benign human disease results from loss-of-function mutations of the connexin 32 gene. This gene encodes a widely expressed connexin or gap-junction-forming protein, with a predicted relative molecular mass of 32K. The disease in question is the X-linked form of Charcot-Marie-Tooth disease (CMTX), a peripheral neuropathy with onset late in childhood. In this disease myelin degenerates, and motor and sensory function are impaired, males being generally more severely affected than females. The big questions raised by the new observation centre on the mechanism by which the myelinating Schwann cells are affected, and why there is no obvious effect in all the other tissues that express connexin 32 (Cx32).

Gap junctions are composed of small aqueous channels that connect the cytoplasmic compartments of the coupled cells³. These channels permit small molecules and ions to pass between cells and mediate electrical and metabolic coupling. Twelve connexin isoforms are known in rodents, each encoded by a separate gene⁴. At least four corresponding connexins occur in humans. All are capable of forming homomeric channels.

Some connexins are found in many tissues; others in only a few. Single cells can express more than one connexin. Heteromeric hemichannels containing more than one connexin type may occur for some combinations; heterotypic junctions can form between some but not all combinations of homomeric hemichannels. Exploration of the functional differences among the connexins in respect to voltage dependence of junction conductance, permeability, and regulation by phosphorylation and transcriptional controls, is a lively area of research⁵.

Schwann cells do in fact express connexin 32 protein, although this was not evident until after the CMTX mutations were identified¹. Gap junctions were known to form between Schwann cells in culture⁶ and *in vivo* during Wallerian degeneration and regeneration⁷, but had not been described in intact nerves. Oligodendrocytes, which form central myelin, make gap junctions with each other and with astrocytes, and also form 'reflexive' junctions between cytoplasmic loops in the perinodal regions and Schmitt-Lantermann incisures^{8,9}. That there are reflexive gap junctions of this kind in Schwann cells is suggested by immunocytochemical staining for Cx32 at the light microscope level¹. These junctions could easily have higher conductance and permeability than the cytoplasmic pathway around the myelin spiral, but the importance to the myelin-forming cell is obscure. Whatever gap junctions do for peripheral

nerve, their loss is more important distally than proximally, and the effects may not be apparent for years.

Of the 15 different CMTX mutations in the coding region of the Cx32 gene, two are truncation mutants that certainly would lead to loss of function^{1,2}. Others are point mutations of highly conserved residues in functionally important regions such as the third membrane-spanning region, which is thought to line the channel, and the extracellular loops which link together in channel formation. A few of the mutations might not cause total loss of coupling, which should be readily testable by expression of the mutant connexins in *Xenopus* oocytes (D. Paul of Harvard University Medical School tells me that he has shown total loss of function in one of the mutants). It will be interesting to see if disease is less severe with mutations that cause partial loss of function. Genetic background may also be important. Two CMTX lineages are wild-type in the Cx32-coding region, and presumably have mutations in upstream regulatory regions.

Connexin 32 is expressed in liver, pancreas, kidney and some neurons³, as well as in myelin-forming cells. Gap junctions in inexcitable tissues are presumed to allow cells to work together. It now becomes of interest to look more carefully at CMTX subjects for subtle effects on such aspects as liver function, particularly when the organ is challenged by metabolic stress or disease. Gap junctions have also been implicated in transmission of developmentally important signals, because they come and go at appropriate times for them to transmit signals and because interference with coupling may disrupt development¹⁰. Where the developmentally significant inducing molecules are peptides that act on extracellular receptors, and are too large to cross gap junctions, an inductive role of gap junctional communication can be rejected. In any case, humans do not require Cx32 for essentially normal development.

Gap junctions have also been said to be involved in tumour suppression. In hepatomas, for example, Cx32 is generally reduced in level and intercellular communication is impaired¹¹. The hypothesis is that coupling allows growth-suppressing molecules to diffuse into initiated cells from neighbouring normal cells, or permits growth-inducing molecules to diffuse out to the neighbours. More growth allows the accumulation of more genotoxic events, progressing ultimately to malignancy. But the lack of reports of an increase in tumours in CMTX subjects is an indication that Cx32 is not an important tumour suppressor in humans.

In carrier (heterozygous) females, about half the Schwann cells should express the defective Cx32, because of X-chromosome inactivation. The grain of the mosaic of X-inactivation in Schwann

Bacterial designs

BACTERIA have developed spectacular ways of coping with hardship. In times of stress, they may exhibit cooperative survival strategies which cause the colony to aggregate into intricate growth patterns such as that shown here. Similar complex growth patterns are well-known for non-living systems — the electrodeposition of metals, for example.



On page 46, Ben-Jacob *et al.* show that the complexity of living systems does not prevent one from being able to develop relatively simple mathematical models of the bacterial growth process using ideas imported from the physical sciences. The key to pattern formation in bacteria, however, is chemotactic communication. Incorporating chemotaxis into the models allows the authors to reproduce the patterns seen experimentally.

Philip Ball

cells and the proximal distal extent have not been determined. In mouse, patchy expression is apparent in cross-sections of nerves from mosaics formed from blastocysts of different myelin mutants¹². Right-left asymmetry in CMTX females has not been reported, suggesting that the mosaic is relatively fine. In the mouse, oligodendrocytes expressing the jimpy mutation die and are replaced by ones expressing the normal gene¹³. Hypomyelination is apparent in the central nervous system of young animals, but disappears with maturation (except in the optic nerve where demyelinated plaques remain). Replacement of mutant Schwann cells by normal cells may be one factor accounting for the lesser severity of CMTX disease in females.

What is Cx32 doing in all the apparently unaffected tissues that express it? Further study of that question would seem justified on clinical grounds alone. And how can one explain the widespread lack of effect of Cx32? One can retreat into the defence of redundancy; the gene product is so important that there are several genes that make equivalent products. Indeed, many cells, including hepatocytes and pancreatic acinar cells, express Cx26 along with Cx32. Because a gene that is unimportant could be easily lost, a more likely explanation is that even small survival advantages provide very strong selection over evolutionarily significant times; this seemingly important gene may indeed be very important, but only in the long run.

Finally, some developments have yet to reach the literature. For instance, a Cx43 mutant in mice is lethal, as described by J. Rossant and G. Kidder at the December meeting of the American Society for Cell Biology. Surprisingly, the embryos survive through term, even though Cx43 is expressed as early as the eight-cell stage¹⁴ and coupling seems to be required for compaction¹⁵. The embryos have malformed hearts, which may be the cause of

death. At the same meeting, S. H. Britz-Cunningham and W. H. Fletcher reported that several cases of human viscerotaxial heterotaxia involving malformation of the heart were associated with mutations of putative phosphorylation sites of Cx43. This is a connexin that is more difficult to get along without. Moreover, in these times of fiscal stringency and focused

research, gap-junction researchers should be better off for working on an organelle that has demonstrated rather than merely hypothesized disease relevance. □

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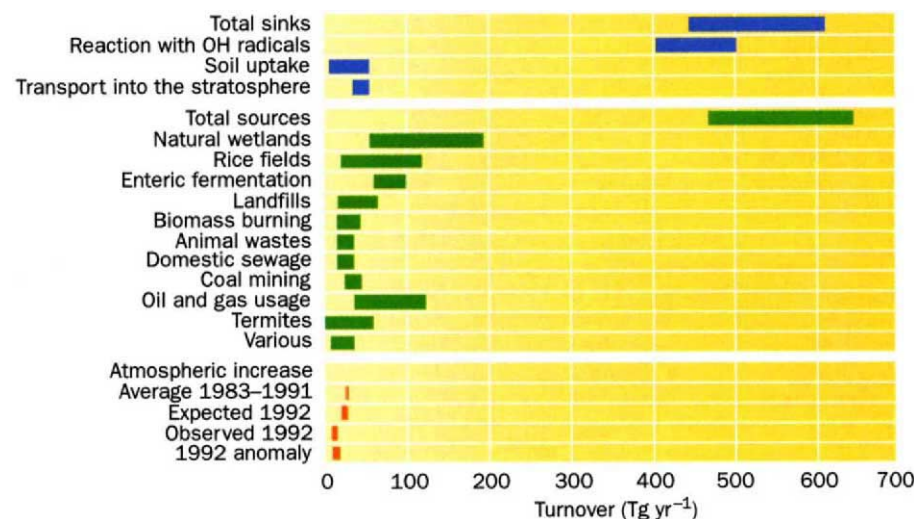
GREENHOUSE GASES

Anomalous methane

J. Rudolph

THE atmospheric concentration of the greenhouse gas methane has more than doubled during the past 200 years, rising over the past 15 years by an average of 1 per cent a year (roughly 17 parts per billion by volume in 1990). But in January this year, Dlugokencky *et al.*¹ reported that the average methane increase worldwide was only 4.7 p.p.b.v. during 1992. In the Northern Hemisphere, the increase was just 1.8 p.p.b.v. What is going on?

p.p.b.v. per year corresponds to a reduction of 9–13 Tg (1 Tg = 10¹² g) in the tropospheric global emission, about 2 per cent of the total annual tropospheric methane turnover (see figure). A change of a few per cent in the strength of one of methane's major sources or sinks might explain the 1992 anomaly. The uncertainties in the budget estimates exceed the magnitude of the missing 1992 methane change by far, so explanations can only be



Comparison of the 1992 methane increase with the tropospheric budget of methane. The horizontal bars indicate the estimated range of uncertainty. The source and sink estimates are adapted from Lelieveld and Crutzen¹⁰.

In fact, the annual increase rate has been gradually slowing down over the past 10 years. From the general trend observed by Khalil and Rasmussen² between 1983 and 1990 we would expect a global increase of 11.3 ± 0.5 p.p.b.v. per year for 1992. A somewhat lower increase of 8.6 ± 0.3 p.p.b.v. per year can be estimated from the results of Steele *et al.*³. Both predictions are well above the 4.7 ± 1 p.p.b.v. per year observed by Dlugokencky *et al.* during 1992.

Between 1983 and 1990 the global annual growth rates deviated by 1–1.5 p.p.b.v. per year or less from the average³, so the lower rate for 1992 certainly suggests unexpected changes in the methane budget. This slowing by 4–6

based on evidence that indicates unusual changes in one of the budget positions.

Dlugokencky *et al.* argue that the rapid economic changes in the former Soviet Union and Eastern Europe could have produced great changes in methane emissions from fossil fuel use. They look at two aspects: decreasing consumption of fossil fuels, and reduced losses during production and distribution of natural gas as a result of efforts to repair leaks in the distribution system. We can use the emission factors and the natural gas production and consumption data for Eastern Europe and the former Soviet Union⁴ to estimate a methane emission rate of 15–35 Tg per year for this region. In the case of a complete conversion to 'Western' stan-

- Bergoffen, J. *et al.* *Science* **262**, 2039–2041 (1993).
- Fairweather, N. *et al.* *Hum. molec. Genet.* **3**, 29–34 (1994).
- Bennett, M. V. L. *et al.* *Neuron* **6**, 305–320 (1991).
- Bennett, M. V. L., Zheng, X. & Sogin, M. L. in *Molecular Evolution of Physiological Processes* (ed. Fambrough, D.) (47th Ann. Symp. Soc. Gen. Physiol. in the press).
- Hall, J. E., Zampighi, G. A. & Davis, R. M. (eds) *Prog. Cell Res.* **3**, 1–333 (1993).
- Chanson, M., Chandross, K. J., Rook, M. B., Kessler, J. A. & Spray, D. C. *J. gen. Physiol.* **104**, 925–946 (1993).
- Tetzlaff, W. *J. Neurocytol.* **11**, 839–858 (1982).
- Sandri, C. & Van Buren, J. M. *Prog. Brain Res.* **46**, 201–265 (1982).
- Schnapp, B. & Mugnaini, E. in *Physiology and Pathobiology of Axons* (ed. Waxman, S. G.) 83–123 (Raven, New York, 1978).
- Warner, A. *Sem. Cell Biol.* **3**, 81–91 (1992).
- Mesnil, M. & Yamasaki, H. *Molec. Carcinogen.* **7**, 14–17 (1993).
- Peterson, A. C. *J. Neurosci.* **5**, 1740–1754 (1985).
- Knapp, P. E., Skoff, R. P. & Redstone, D. W. *J. Neurosci.* **6**, 2813–2822 (1986).
- Valdimarsson, G., De Sousa, P. A., Beyer, E. C., Paul, D. L. & Kidder, G. M. *Molec. Reprod. Dev.* **30**, 18–26 (1991).
- Becker, D. L., Leclerc-David, C. & Warner, A. *Development suppl.* 113–118 (1992).

dards (European or North American emission factors) the methane emissions would decrease to 2.6–6 Tg per year. So the reduction potential of 13–29 Tg per year is enough to explain a decrease of 6–13 p.p.b.v. per year, roughly twice the observed 1992 anomaly.

But how realistic is this? It seems unlikely that half the natural gas production and distribution systems could have been transformed in less than a year. Reductions in gas and coal production in the former Soviet Union, too, can only have had a minor impact on the atmospheric methane budget. The methane production decreased between 1990 and 1993 from $814.5 \times 10^9 \text{ m}^3$ to $780 \times 10^9 \text{ m}^3$ (ref. 5). This corresponds to a reduction in methane emissions of 0.6–1.5 Tg per year. The total emission of methane from coal mining in the former Soviet Union is estimated to be 7.9 Tg per year (ref. 6), so an extreme reduction would be needed before we saw significant consequences for the atmospheric methane budget.

Let us look at other possible explanations for the low growth rate. As the methane increase in the Northern Hemisphere was particularly low (1.8 ± 1.6 p.p.b.v. per year) and that in the Southern Hemisphere was very close to our expectations (7.7 ± 1 p.p.b.v. per year), Dlugokencky *et al.* argue for an effect specific to the Northern Hemisphere. Since 1983, though, the methane increase rates in the two hemispheres taken individually deviated several times by 3–5 p.p.b.v. per year from the average trend³. Fluctuations in one hemisphere were anti-correlated with changes in the other. Steele *et al.*³ attribute this to variations in the exchange rate between the two hemispheres: as about half the methane that is removed from the atmosphere in the Southern Hemisphere was originally imported from the Northern Hemisphere, a minor variation in the interhemispheric exchange rate can change the distribution of the methane accumulation substantially. Therefore we cannot limit our search to the Northern Hemisphere.

There is some evidence that biomass burning in the tropics was less intensive in 1992 than in previous years. For example, the burnt area in the Kruger National Park was less than one-third of the area burnt annually between 1989 and 1991 (W. Trollop, personal communication). It is difficult to estimate the possible reduction worldwide, but the total tropical methane emissions from biomass burning are around 26 Tg per year including 10 Tg per year from deforestation and wood burnt as fuel⁷. Again, an extreme change would be needed to explain the whole of the 1992 methane anomaly.

Another possible culprit is the eruption of Mount Pinatubo in 1991. One of its effects on climate was to reduce the average temperature in the lower troposphere

of the Northern Hemisphere in 1992 by 0.7 K. The cooling could have reduced methane emissions from natural wetlands, but as Dlugokencky *et al.* point out, this can only partly explain the 1992 methane anomaly. Just how changes in rainfall and evaporation rates would affect methane emissions from wetlands is difficult to quantify and therefore extremely uncertain. A change in tropospheric temperature would also change the rate at which methane is removed there. From the temperature dependence of the reaction rate constant for CH_4 with OH (ref. 8), it can be calculated that a decrease of 0.5 K would actually reduce the chemical methane sink by about 1 per cent, corresponding to an increase of 4 Tg per year, provided the OH radical concentration does not change. So should we be looking for an even larger anomaly in the methane sources for 1992? Unfortunately we know very little about changes of the tropospheric OH radical concentrations in 1992. As the removal by this reaction is by far the largest item in the global methane budget, minor changes in the average tropospheric OH radical concentration could easily explain the reduced methane growth rate.

All in all, although we can identify several sources that might have been lower in 1992, the evidence is not conclusive. Obviously, the future development of the tropospheric methane trend will allow us to test some of these speculative explanations. Further clues should come from measurements of the isotopic composition of methane, which are being made at several monitoring stations.

A final thought. David Keeling's atmospheric carbon dioxide record at the Mauna Loa monitoring station also showed an anomalously low increase in 1992 (see for example the News and Views by Sarmiento⁹). This might be a coincidence, but it might indicate changes in factors that influence atmospheric methane and carbon dioxide in a similar way. □

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1. Dlugokencky, E. J. *et al.* *Geophys. Res. Lett.* **21**, 45–48 (1994).
2. Khalil, M. A. K. & Rasmussen, R. A. *Chemosphere* **26**, 803–814 (1993).
3. Steele, L. P. *et al.* *Nature* **358**, 313–316 (1992).
4. Craig, E., Picard, D., Pope, P. & Rosland, A. in *Proc. int. IPCC Workshop on Methane and Nitrous Oxide* (ed. van Amstel, A. R.) 41–61 (RIVM Report No. 481507003, Bilthoven, 1993).
5. Sedykh, A. *Oil and Gas: Russia, Central Asia and the Caucasus* **2**, 59–68 (1993).
6. Kirchgessner, D. A., Piccot, S. D. & Winkler, D. J. *Chemosphere* **26**, 453–472 (1993).
7. Hao, W. M. & Ward, D. E. *J. geophys. Res.* **98**, 20657–20661 (1993).
8. Vaghjani, G. L. & Ravishankara, A. R. *Nature* **350**, 406–409 (1991).
9. Sarmiento, J. L. *Nature* **365**, 697–698 (1993).
10. Lelieveld, J. & Crutzen, P. J. in *Proc. int. IPCC Workshop on Methane and Nitrous Oxide* (ed. van Amstel, A. R.) 17–25 (RIVM Report No. 481507003, Bilthoven, 1993).

DAEDALUS

High power

BACK in the 1970s technomaniac projects such as nuclear power stations were still in vogue. Environmentalists argued that solar power seemed a far safer, cheaper and reassuringly low-tech power source. The technomaniacs, fearing that they were losing this argument, sought to hijack solar power themselves. They proposed an enormously expensive and complicated solar-power scheme: a system of geosynchronous satellites equipped with huge solar collectors, which would transform solar energy to microwaves and beam them down to vast aerial-farms on the ground. A satellite is always above the clouds, of course, and its descending microwave beam would penetrate them without loss. (The beam could also be steered and aimed as a space weapon, though the technomaniacs kept quiet about this). Daedalus now has a much simpler elevated solar-power scheme.

He plans to lift solar panels above the clouds on ordinary hydrogen or helium balloons. Each transparent balloon is internally silvered on one hemisphere, whose concave reflecting surface is kept pointing towards the Sun. Sunlight is thus focused on a region inside the balloon, which is occupied by a bank of photovoltaic cells. Illuminated so intensely, they can be smaller and lighter for a given power output than a normal array. The extreme cold 5 or 10 kilometres up prevents the cells from overheating. Furthermore, the waste heat they reject into the balloon expands its internal gas and increases its lift.

At first Daedalus planned to anchor his balloons with carbon-fibre cables braided with aluminium, down which their current would travel to the grid distribution system. The light, strong carbon fibre could easily absorb surplus lift and resist sideways wind loads. But 5 or 10 kilometres of aluminium power-cable would be far too heavy for the balloon to lift. So Daedalus now plans to use microwave transmission. He will beam the power to Earth down parallel metallized cables configured as a 'lecher-line' waveguide. The beam will thus descend without spreading out; its energy will travel mainly through air, not metal; and elaborate and heavy aerials at both ends will still be avoided.

Sadly, this elegant scheme has a serious drawback compared to the satellite project. Even on the night side of the Earth, a geosynchronous satellite can still see the Sun; but Daedalus's balloons will lose power at night. They will not be able to supply electricity for its one really important large-scale use: electric light. They will only supplement, not replace, existing power sources. David Jones

Avalanche survival chances

SIR — The risk of triggering an avalanche makes ski touring the most dangerous winter sport, claiming about 150 lives annually in the Alps alone¹. Using data² on all avalanche disasters in Switzerland from 1981 to 1991, we have calculated survival probability in relation to the length of time buried under the snow. At 15 min the survival probability (92%) is markedly higher than previously assumed, but the survival function then drops precipitously to only 30% at 35 min, representing deaths through acute asphyxiation. Thereafter, survival is impossible without an air pocket. After 90 min, victims gradually succumb to hypoxia and hypothermia unless the air pocket is open to the outside. This reassessment of survival probability has far-reaching implications for recommended rescue strategies, emphasizing the importance of rapid and efficient help by uninjured companions and explaining the low success rate achieved by organized rescue parties.

We have analysed precise, minuted rescue data². Of 422 buried skiers, 241 (57%) were dead on extrication. The mean depth of burial under the snow (head) was 105 ± 85 cm (s.d.). An analysis of the relationship between rescue outcome, depth of burial and time of extrication (data not shown) indicates that there is no direct influence of depth of burial on survival, and so the poor results on extrication of deeply-buried skiers merely reflect the generally prolonged rescue time involved.

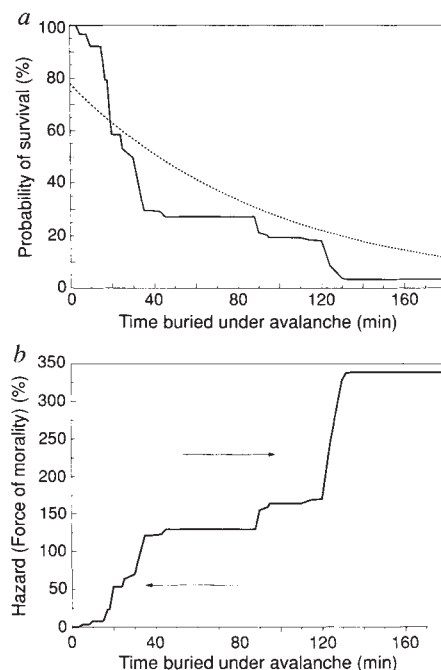
All rescue directives to date are based on the survival function proposed by Schild³. But Brugger and Falk's⁴ computer-assisted procedure⁵ applied to the avalanche rescue data² allows estimation of survival probability with far greater accuracy. The fundamental difference in survival function (*a* in the figure) lies in the steep drop of the present curve at 15 min until 35 min, with a further dip commencing at about 90 min, as compared with the gradual exponential decrease in survival probability assumed by Schild.

The only finding giving grounds for optimism is that the initial survival probability is much higher than previously assumed. Indeed, of the 123 skiers extricated within 15 min, only 8 were dead and, moreover, only 2 had died of asphyxia (extrication times 10 and 15 min), whereas the remaining 6 skiers had all sustained fatal injuries during descent of the avalanche. The survival probability then plummets from 92% at 15 min to only 30% at 35 min, in contrast to the hitherto-accepted gentle decrease from 67 to 55% (ref. 3) over the same period. The corresponding pronounced increase in cumulative hazard function (*b* in the figure) is comparable

with the well-known observation of rapid decline in probability of successfully resuscitating acutely asphyxiated patients. This fatal drop in survival function probably results from acute asphyxiation of all victims without an air pocket.

The virtually constant survival (*a* in the figure) and cumulative hazard (*b*) functions between 35 and 90 min indicate that for skiers still alive in a fortuitous or self-created air pocket at the beginning of this phase, the risk of dying is minimal for the next 55 min. It is known that the snow cover prevents rapid hypothermia (maximally 3 °C per hour)⁶ and that oxygen consumption decreases significantly with lowering of the body temperature and loss of consciousness⁷.

The survival probability then falls from 27% at 90 min to only 3% at 130 min (*a* in the figure), mirrored by the increase in hazard function (*b*). Hence, victims with a



a, Survival function and *b*, cumulative hazard function, of totally buried avalanche victims, 1981–91. The survival function (solid line) is shown in comparison with the hitherto-accepted function proposed in ref. 3 (dashed line). Both show the cumulative probability of survival under the snow in relation to time since burial. Our assessment of the survival function was based on a non-parametric estimation procedure in ref. 5, whereby the extrication times are entered as double-censored data (dead: left censored; alive: right censored). *b*, The cumulative hazard function, $-\log(\text{survival function})$, shows the force of mortality in relation to duration of burial. The two phases of increase in cumulative hazard function are indicated by arrows and represent the prognosis for groups without (lower arrow) and with an air pocket (upper arrow).

'closed' air pocket eventually succumb between 90 and 130 min after descent of the avalanche. Death is due to a combination of "slow asphyxia" and hypothermia. (The decrease in body core temperature appears to be greatly slowed down if oxygenation is adequate, especially with an 'open' air pocket.) Thus, in the absence of fatal injuries, speed of extrication from the avalanche and existence of an air pocket are the decisive factors determining survival. The fact that there was no decline in the annual mortality rate between 1981 and 1991, despite increasing standards of professional rescue techniques and medical emergency back-up services, is largely explained by the difficulties in mobilizing mountain rescue teams within the brief optimum survival time.

Reduction of the present high mortality rate depends on increasing the proportion of skiers freed within 15 min, which, realistically, means by uninjured companions. Professional help should be sought immediately after, but not during, this critical phase. We believe that mountaineering organizations should teach skiers mandatory safety precautions, as well as the basic techniques of finding, extracting and resuscitating avalanche victims. At present, many skiers carrying search appliances (transceivers) are insufficiently familiar with their use, with fatal consequences. Another essential step would be to develop self-help techniques to facilitate creation of a life-saving air pocket, which would give the skier a relatively safe haven for about 90 min which is the new time goal set for professional rescue. However, optimal equipment and experience provide no life guarantee, and may induce a false sense of security. Prophylaxis remains the only reliable safeguard for the individual.

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- Valla, F. *Bull. Int. Comm. Alpine Rescue* (ed. Schori, M.) (Birchwil, Switzerland, in the press).
- Etter, H. J. et al. *Durch Lawinen verursachte Unfälle und Schäden im Gebiet der Schweizer Alpen, Winterberichte des SLF 46-55 (1981-1991)* (Eidgenössisches Institut für Schnee und Lawinenforschung Davos).
- Schild, M. *Lawinen, Ortung-Phylaxe-Rettung* 56-84 (Fond. Int. Vanni Eigenmann, Milano, 1975).
- Brugger, H. & Falk, M. *Wien. klin. Wschr.* **104**, 167-173 (1992).
- Turnbull, B. W. *J. R. statist. Soc. Ser. B* **38**, 290-295 (1976).
- Locher, Th., Walpoth, B., Pfluger, D. & Althaus, U. *Schweiz. med. Wschr.* **121**, 1020-1028 (1991).
- Hossli, G. *Lawinen* 77-87 (Juris, Zürich, 1976).

Controlling nanostructures

SIR — Röder *et al.*¹ report nanometre-scale structures built by deposition of diffusing particles that aggregate on surfaces. We have developed a microscopic model that mimics the same process, and produces morphologies that remarkably resemble the experimental structures.

The model is defined as follows: (1) Deposition. Particles are deposited at randomly-chosen positions of the surface at a flux F per lattice site per unit time. (2) Diffusion. A cluster of connected particles is chosen at random and moved north, east, south or west by one lattice constant per unit time with a probability proportional to its mobility, which is given by $D_s = D_1 s^{-\gamma}$, where s is the number of particles in the cluster, D_1 is the diffusion constant of the monomers and γ characterizes how the mobility of a cluster depends on its size. (3) Aggregation. If two particles come to occupy neighbouring sites, they stick irreversibly.

The model can be tested by explicit comparison with the experimental data in ref. 1. There are no free parameters provided we introduce the experimental values for the flux and the diffusion constant. The diffusion constant of the monomers is given by $D_1(T) = D_0 \exp(-E_d/kT)$ with $E_d = 0.14$ eV (ref. 1) and $D_0 = 5 \times 10^{11}$ (ref. 2). Using the experimental values of the fluxes, we find $F/D_1 = 10^3$ corresponds to Fig. 1a of ref. 1, and $F/D_1 = 10^{-10}$ to Fig. 1d. The figures here show results of the model with these flux values; note that the morphologies compare well with Fig. 1a and d of ref. 1.

In general, our model allows one to distinguish the effects of deposition, diffusion and aggregation. We find that tuning

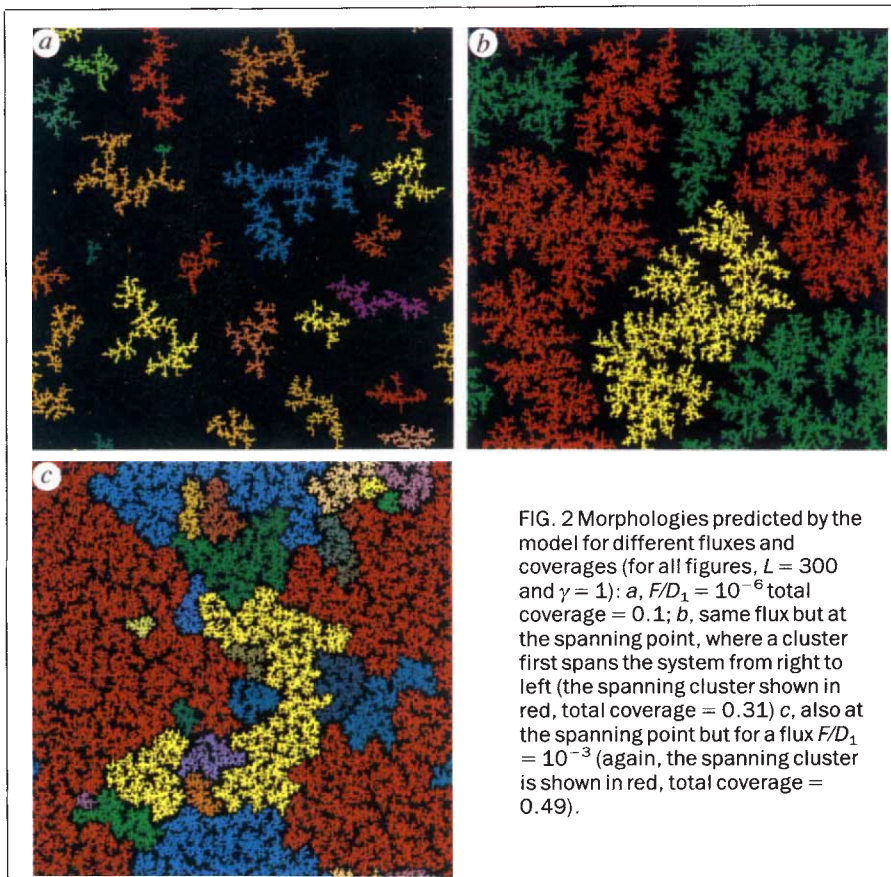


FIG. 2 Morphologies predicted by the model for different fluxes and coverages (for all figures, $L = 300$ and $\gamma = 1$): a, $F/D_1 = 10^{-6}$ total coverage = 0.1; b, same flux but at the spanning point, where a cluster first spans the system from right to left (the spanning cluster shown in red, total coverage = 0.31) c, also at the spanning point but for a flux $F/D_1 = 10^{-3}$ (again, the spanning cluster is shown in red, total coverage = 0.49).

the relative strength of, for example, deposition and diffusion, generates a rich range of morphologies — including diffusion limited aggregation (DLA), cluster-cluster aggregation, and percolation³. The length- and timescales characterizing these morphologies depend on experimentally controlled parameters like deposition flux and diffusion constant, raising the possibility that the model could be used for a controlled design of nonstructure morphologies. Indeed, the model

makes specific predictions, for example that the typical size of the DLA-like structures scales as $(F/D_1)^{-1/4}$.

Varying the model parameters produces a rich range of morphologies (Fig. 2), and the model may be useful in a variety of situations where diffusion and aggregation occur in the presence of continuous deposition. The model was originally motivated by thin-film deposition experiments in which not isolated atoms but rather aggregates (compact, spherical molecules of about 5-nm diameter, containing about 2,000 atoms) are deposited on a surface⁴. The morphologies of Figs 1 and 2 also resemble experimental images obtained by such low-energy cluster beam deposition experiments on substrates maintained at low temperatures (compare Fig. 2a to Fig. 3 of ref. 4).

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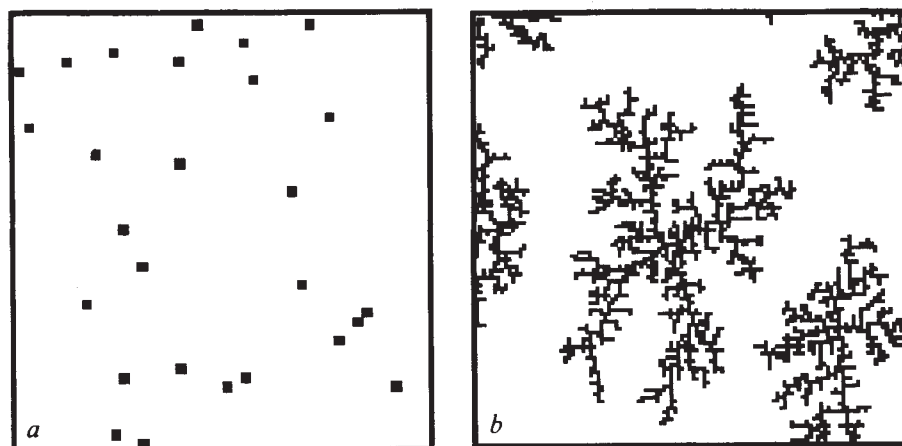


FIG. 1 Morphologies obtained in the present model for two different values of F , D_1 and total surface coverage all chosen to correspond to the experimental parameters used in obtaining the data shown in Fig. 1a and d of ref. 1. a, $F/D_1 = 10^3$, and total coverage of 0.012. b, $F/D_1 = 10^{-10}$, and total coverage of 0.12. The simulation lattice had 200×200 sites; the portion shown here corresponds to Fig. 1a and d of ref. 1, which are also a portion of the total experimental system. We set $\gamma = 10$ (large clusters rarely move — J. P. Bucher, personal communication).

1. Röder, H., Hahn, E., Brune, H., Bucher, J. P. & Kern, K. *Nature* **366**, 141–143 (1993).
2. Venables, J. A., Spiller, G. D. T. & Hanbücken, M. *Rep. Prog. Phys.* **47**, 399–459 (1984).
3. Vicsek, T. *Fractal Growth Phenomena*, 2nd edn. (World Scientific, Singapore, 1992).
4. Jensen, P. *et al. Physica A* **185**, 104–110 (1992).

Fields of influence

David Cahan

Robert Mayer and the Conservation of Energy. By Kenneth L. Caneva. Princeton University Press: 1993. Pp. 439. \$49.50, £33.

DURING the 1840s, several scientists and physicians — among them Robert Mayer, Ludvig Colding, James Prescott Joule and Hermann von Helmholtz — proposed what they, or their successors, called the principle of the conservation of force (or energy). By the 1850s there were already disputes as to who most merited the appellation of discoverer or co-discoverer of the principle. As the title of Kenneth Caneva's scholarly and perceptive work implies, historians of science are still assessing the merits of the pretenders to the crown. Yet they now do so less, if at all, to determine the discoverers than to understand the evolving meaning of such terms as 'force' and 'energy', the early thinking about thermodynamics and physiology, and the more general growth of the modern scientific disciplines.

Caneva greatly advances our understanding of Mayer's work on the conservation of energy — a phrase Mayer never used — by analysing it in the light of its broader scientific, medical, philosophical and theological contexts. The book is therefore not only a study of the slow, complex evolution of Mayer's thinking and how Mayer may have clarified to himself the meaning of what he had discovered. It is also a study of physiology and, to a lesser extent, physics and chemistry in Germany during the 1830s and early 1840s, and of the sources that Mayer may have consulted during his intellectual development.

Without seeking to offer a biography of Mayer (1814–78), Caneva presents a brief, integrated account of Mayer's life and work. We are reminded, for example, of his youthful experience in trying to construct a *perpetuum mobile*, and learn much about his medical training at the University of Tübingen in the 1830s, and his voyage to the Dutch East Indies in 1840, where he studied the physiology of the blood and observed that the blood of Europeans there was lighter in colour than expected. Here too are details of Mayer's religious upbringing and beliefs, in particular his faith in a providential and benevolent God who created a harmonious world and his adamant opposition to materialism. These and other aspects of

Mayer's life are recounted with an eye to assessing their possible contribution to the evolution of his scientific work. Above all, Caneva shows that at the heart of Mayer's efforts and claim to be the discoverer of conservation of force lay his attempt to show numerically the equivalence of heat and motion. Although Mayer thought there was a causal (force) relationship between the two, he did not reduce heat to motion. For Mayer, forces were indes-

IMAGE
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REASONS

Julius Robert Mayer.

tractible, mutable and immaterial; they were not properties of matter, but rather the independent, imponderable and analogous counterparts to matter. Force was to physics what matter was to chemistry: the foundation of the entire subject.

Caneva treats in great, indeed perhaps too great, detail the physiological, medical, physical and chemical contexts of Mayer's work and the general topics of contemporary German views on the nature and scope of science, as well as the religious and spiritual climate in which Mayer worked. We learn about the probable influence on Mayer of studies of blood, respiration and animal heat; the gradual acceptance of the exclusive treatment of physiological phenomena in chemical and physical terms; the slow acceptance of energetic transformations;

the relationship of an organism's vital processes to external physical and chemical processes; the alleged role of so-called vital forces in an organism's activity; various analogies and metaphors (force and matter, machines as organisms, the Solar System and living organisms); Mayer's thoroughgoing opposition to materialism; and even homeopathy.

Although his understanding of contemporary physical thought was weak, Mayer nevertheless brought about a central innovation in physics by breaking with the contemporary understanding of force: he made the concept independent of matter and gave it a numerical measure, ultimately calculating the numerical equivalence between heat and work. Scientists and physicians such as J. H. F. Autenrieth, Jacob Friedrich Fries, Antoine Lavoisier,

Justus Liebig, Johannes Müller, Theodor Schwann and Friedrich Tiedemann constituted the intellectual milieu in which Mayer worked and probably influenced him. Caneva also argues that contemporary philosophical and theological issues shaped both Mayer's notion of the character and scope of science and his central concept of force: for example, the ideas that causes are linked to effects, which in turn are to be represented as natural forces, and that the scope of science is limited to explaining physical phenomena, so that entities such as souls, minds and vital forces were none of Mayer's strictly scientific business.

Caneva concludes by seeking to reconstruct Mayer's work in context, both by uniting the earlier parts of the book and by assessing the supposed influence of *Naturphilosophie*. He analyses in detail Mayer's publications in their contemporary setting, tracing the probable evolution of Mayer's thought from his surprising observation in the Dutch East Indies, to his new concept of force, and on to the mechanical equivalence of heat. By 1845 Mayer had achieved what he called "the mechanical theory of heat". Certain motifs and scholarly claims notwithstanding, there is little if any evidence that *Naturphilosophie* played any part in Mayer's thinking.

Caneva's meticulous, critical and impressive examination of Mayer's probable route to energy conservation should be of interest to all students of nineteenth-century science. □

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Missing rings

H. Jay Melosh

The Geology of Multi-Ring Impact Basins: The Moon and Other Planets. By Paul D. Spudis. Cambridge University Press: 1993. Pp. 263. £37.50, \$59.95.

MODERN studies of the Moon, culminating in the manned Apollo landings of the late 1960s and early 1970s, revealed that most lunar landforms were created by the impact of high-speed meteoroids. Even the broad volcanic plains fill depressions created by impacts. The lunar surface is covered with impact craters at all scales, from only a few microns in diameter up to vast structures that may occupy an entire hemisphere. Not surprisingly, the biggest impacts have had the most profound effect. The fundamental geological fabric of the Moon was established by several dozen huge impacts that deeply shattered its crust and spewed ejecta over millions of square kilometres of its surface. The circular scars of these impacts range in diameter from roughly 300 km up to the recently confirmed South Pole/Aitken basin with a diameter of 2,600 km, larger than the Moon's radius.

These great structures are not simply scaled-up versions of smaller craters, however, but show their own peculiarities and so are given the special name 'basins' by lunar geologists. The most obvious difference between a crater and a basin is the presence of two or more roughly circular rings around a low-lying central plain. The importance of the concentric structures was first recognized for the Orientale basin by W. K. Hartmann and G. P. Kuiper in 1962 and the feature has since become the defining characteristic of these large structures on the Moon. Impact basins are not, however, confined to the Moon, but have been recognized on all the terrestrial planets (including Earth) and are seen in modified form on the icy satellites Ganymede and Callisto.

Although multi-ring impact basins are a widespread and distinctive element of planetary crusts, the mechanism by which the rings themselves form is still highly controversial. Even the identification of which ring (if any) corresponds to the edge of the cavity excavated by the impact is still contentious: a few planetary scientists suppose that the outermost ring is the rim of this cavity, while most suppose that at least some of the rings formed outside the cavity during its collapse. Another unexplained aspect of multi-ring basins is that the diameters of adjacent rings often have ratios roughly approximating $\sqrt{2}$ or 2, which has led some planetary geologists to an almost mystical insistence that the rings are spaced by intervals of $\sqrt{2}$ (ring diameter ratios of 2 are therefore some-

times explained by a 'missing' ring spaced at the 'correct' interval).

The Geology of Multi-Ring Impact Basins is the first book-length treatment of this subject. The author attempts to bring together the wealth of geological, geophysical, geochemical and remote-sensing data that have been collected about basins and to address the major problems of basin formation. The emphasis is strongly lunar: basins on other planets and satellites are treated in a single chapter, whereas each of five lunar near-side basins is given its own chapter, with a sixth chapter devoted to the processes of their formation. Each of the chapters on the five lunar basins describes the structure and geology of the basin, presents the results of remote spectral reflectance studies and, where appropriate, discusses the implications of the Apollo or Luna lander samples for basin excavation. These chapters succeed in collecting a great deal of otherwise disparate data and give a valuable overview of the geology of the lunar nearside.

In other ways, however, the book is seriously flawed. The author cites the existence of up to six concentric rings around many basins, all perfectly circular and spaced at intervals of exactly $\sqrt{2}$. Most planetary mappers simply cannot see this many rings, a fact that is supported by the US Geological Survey series of planetary maps, which do not show most of the rings Spudis illustrates in his book. Furthermore, it is obvious that many of



FOLLOWING their exploration of the Amazon, Portuguese traders, as shown here, gained considerable control of their new-found lands in the early seventeenth century. This engraving is taken from *Explorers of the Amazon* by Anthony Smith, now out in paperback (Chicago University Press, \$15.95, £12.75). For a review, see *Nature* 344, 901 (1990).

the rings are not perfectly circular (Spudis himself states this in describing the western portion of the Orientale system). Nevertheless, ring diameters listed in the book are presented without uncertainties or ranges. Spudis raises the rough $\sqrt{2}$ ring spacing to an apparent law of nature, although the reader must take his word for the 'correct' ring diameters to obtain the near-perfect fit that he describes. One has to suspect that Spudis searches for rings by looking for isolated topographical features along circles spaced at the expected intervals and, not surprisingly, usually finds them.

There are several other less important problems with the text. Jargon and acronyms are used too frequently (the term "KREEP" is not defined anywhere), and the book contains many implicit, undefended assumptions (for example, "more mafic" is equated to "deeper origin"). Conflicting views, such as the existence of a Procellarum basin, are often dismissed without substantial discussion. Too many citations refer to unpublished work, mainly by Spudis and colleagues.

Overall, the book does offer a valuable collection of data on multi-ring basins, but any reader should be aware that it is a highly idiosyncratic view of the subject that fails to reflect the mainstream ideas of planetary scientists. □

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Live and let live

Beverly E. Griffin

Emerging Viruses. Edited by S. S. Morse. Oxford University Press: 1993. Pp. 317. £32.50, \$39.95.

A RECENT leading article in *The Times* of London (5 February 1994) attributes to Sir Peter Medawar the description of a virus as "bad news wrapped in protein". But is there no such thing as a good virus? The answer depends on the relationship of an individual to the viral host, for viruses are complete parasites and depend entirely for their existence on their host. If, for example, the host is deemed to be bad, then one's attitude might be altered in favour of the virus. It is this philosophy that has led to the examination of a role for insect viruses as 'insecticides', protecting valuable crops from being destroyed. So, because every living species has its own battery of viruses, from man's point of view there can be such things as good viruses. But by and large viruses are 'bad news' for us; it is from this point of view that *Emerging Viruses* is written.

The basic message of the book is that although many viruses may adopt largely passive roles in their native host, this passivity can be perturbed in a number of ways. The routes considered involve moving the virus via its host to a new environment, one formerly alien to the virus, or the passage of a virus from its natural host to a new species. In both routes, a delicate balance is disturbed, and the virus may become a deadly enemy. Adaptation to the host is part of the life cycle of any virus, but this is a process that requires time (decades or longer).

Last year in *Nature* (364, 201; 1993) I reviewed a book by a US journalist about emerging viruses (*A Dancing Matrix: Voyages Along the Viral Frontier* by R. M. Henig, Knopf, 1993). This was, on ba-

lance, a readable and stimulating book. Henig made frequent references to conversations with S. S. Morse, a scientist at the Rockefeller University and editor of *Emerging Viruses*. It was clear that, to Henig, Morse was a sort of guru with whom she checked her ideas. Despite its occasional technical flaws, Henig's book covered a great deal of the territory now covered by Morse, and often better. *Emerging Viruses* suffers by being a badly edited multi-authored volume, the whole distinctly poorer than the sum of its parts.

The book had its origins in a conference held in Washington DC in May 1989, although it contains additional and updated contributions. There are 28 chapters and 45 authors, most of them from US institutions. (That is in itself not unreasonable: with the exception perhaps of the Pasteur Institute in France and the Ivanovsky Institute of the former Soviet Union, little attention outside the United States has unfortunately been paid to the subject as it relates to humans). Individual chapters are rather artificially grouped under topics that supposedly define them. But the book as a whole impresses one more as a random-chapter stapling job than a coherent whole.

Having said this, I would have to agree with the editor and contributors that where the field of viruses is concerned, we cannot afford to be complacent, so a book covering the topic of the changing patterns of viruses and the emergence of unknown (not necessarily new) ones deserves to be written and carefully read. This point is strongly and convincingly made in chapter 27 by Donald A. Henderson of the US Office of Science and Technology Policy. (Why does this splendid chapter not appear earlier?) To quote him:

The recent emergence of AIDS and dengue hemorrhagic infections, among others, are serving usefully to disturb our ill-founded complacency about infectious diseases. Such complacency has prevailed in this country (USA) throughout much of my career. . . It is evident now, as it should have been then, that mutation and change are facts of nature, that the world is increasingly interdependent, and that human health and survival will be challenged, ad infinitum, by new and mutant microbes, with unpredictable pathophysiological manifestations. . . How are we to detect these at an early date so as to be able to devise appropriate preventive and therapeutic modalities? What do we look for? What types of surveillance and reporting systems can one devise?

Virology books can generally be divided into two types: those that deal with what viruses are, and those that deal with what they do. *Emerging Viruses* falls into neither of these categories. It is a specialist book for specialists, dealing almost exclusively with the so-called RNA viruses, that is, those viruses whose genetic information is carried in RNA, not DNA. These

are the viruses whose rate of mutation is sufficiently fast that controlling them, or predicting their behaviour, is well-nigh impossible. The most widely studied and to some extent the most important at the moment are influenza and HIV, considered by several contributors. Among the numerous provocative and well-written chapters on RNA viruses, there is an excellent one by J. J. Holland that deals biologically and mathematically with, on the one hand, the extreme genetic variability of RNA viruses and, on the other, the most plausible explanations for why DNA viruses are not equally plastic. Variability, or mutation, is an exceedingly important topic for any discipline focusing on intervention, and Holland emphasizes how little we really know about 'natural' mutation and in particular why it can vary so dramatically among different RNA viruses or within the genome of any one of them. High mutation rate means that no RNA virus population is a single entity, but is rather (in Manfred Eigen's term) a "quasi-species".

Emerging Viruses mainly deals with human viruses, or viruses that have recently crossed over into man from other species. In one of the few chapters on the more stable DNA viruses, Frank Fenner, the Australian doyen of virology, discusses a pox virus that is indigenous to several species of monkeys and can also be carried by squirrels. 'Human monkey pox', as it is called, can produce symptoms in unvaccinated humans similar to those produced by smallpox. Following the World Health Organization (WHO) smallpox eradication scheme, and subsequent discontinuation of vaccination, 386 cases of monkey pox in Zaïre (up to 1986) were identified. This is an illustration of a virus menacingly changing its natural host range, a phenomenon more prevalent in, but not restricted to, RNA viruses. Vaccines against smallpox are, indeed, effective against human monkey pox, but the WHO programme has ended. There is one chapter devoted to plant viruses — can or do these ever cross over to man? — and another to the seal virus plague that appeared spontaneously and dramatically in northern Europe in 1988.

Much of the book, however, focuses on the new human diseases that have come (to the United States) from tropical countries, or arisen following man-made alterations in the ecosystem. Some of these outbreaks may indeed be attributable to our naivety about viruses. Outbursts are not necessarily new phenomena, as Howard Temin notes in the introduction to his chapter on retroviruses: sailing ships brought yellow fever from Africa to the Americas in the seventeenth and eighteenth centuries, the disruptions of the First World War coincided with the great influenza pandemic, and so on. Temin and others, in considering the vast

New in paperback

Nuclear Choices: A Citizen's Guide to Nuclear Technology by Richard Wolfson (revised edn). Reviewed in *Nature* 353, 222 (1991). MIT Press, £17.95, \$29.95.

The Space Telescope: A Study of NASA, Science, Technology and Politics by Robert W. Smith. Now with a new afterword detailing the project's problems since its 1990 launch, but written before the recent successful mission to put Hubble into focus. CUP, £16.95, \$24.95.

The Bamboos by F. A. McClure, now with a new foreword and introduction. A classic of botany and horticulture, first published in 1966. Smithsonian Institution Press, \$16.95.

changes in urbanization, population explosions in parts of the world, altering life styles (including jet travel) and so on, wonder how we have escaped with so few pandemics. Again and again, the authors argue that we are at the mercy of viruses, not the other way around. That does not mean we should be defeatist, and I suspect that many of the contributors are concerned with seeing that we are not. But individuals can do little. It requires surveillance at government levels, which means money invested in epidemiology, tropical medicine and communication. Outside the United States, who at high level is considering such problems? Will it prove a regrettable mistake that the UK Medical Research Council closed down its Tropical Medicine Board a few years ago?

Government safety inspectors in Birmingham (England) monitored closely by interested, but not very knowledgeable, scientific correspondents in the daily press, have recently been concerned with what is viewed as a breach of safety rules

involving genetic manipulation of a viral component (see *Nature* 367, 499; 1994). One research laboratory of the university has apparently been closed. *The Times* (op. cit.) used this incident for an attack on academic freedom, censoring scientists for "still inhabiting the sequestered world described by C. P. Snow". Although there may be some justification in this criticism, it seems equally imperative to pay attention to the fact that viruses are manipulated constantly in nature, in a manner well beyond our control and on a scale that transcends many times the efforts of a single laboratory. Let us hope, globally, that our various Health and Safety Executives, and our press, are alert to this fact. How many of them will read this book? May I close by drawing it to their attention? □

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A physicist's tour of chaos

Paul Glendinning

Chaos in Dynamical Systems. By Edward Ott. Cambridge University Press: 1993. Pp. 385. £45.00, \$69.95 (hbk); £16.95, \$29.95 (pbk).

THE term 'chaos' was first used in the context of dynamical systems by T. Y. Li and J. A. Yorke in 1975 (*Am. Math. Monthly* 82, 985–992). Ever since, the group at the University of Maryland (Yorke, C. Grebogi, E. Ott and others) has been at the forefront of work on applied dynamical systems. Ott's book collects together many of the results obtained at Maryland (and a few from other sources) to provide a stimulating selection of topics that could be taught *à la carte* in postgraduate courses. The book is given unity by a preoccupation with scaling arguments, but covers almost all aspects of the subject (dimensions of strange attractors, transitions to chaos, thermodynamic formalism, scattering, quantum chaos and so on).

The study of chaos, and dynamical systems in general, has always been multidisciplinary. Ott approaches mathematical phenomena as a physicist, using the computer as his experimental tool. There is little attempt to make statements mathematically precise and, as in many physical textbooks, the reader is expected to acquire a great deal of knowledge on trust. For example, by looking at computer simulations we are shown that the attractor for the Hénon map, a simple map of the plane to itself, is geometrically complicated ('fractal') and "hence [that

the map] has a strange attractor". The fact that this is not an established result for Ott's example goes unmentioned. (It is only very recently that rigorous results have been obtained on the existence of strange attractors for *some* Hénon maps and these serve only to demonstrate how many other complications are possible.) Nevertheless, his description provides an excellent, intuitive account of chaos, which is essential to developing scientists — much more important than the mathematical niceties that have been swept under the carpet.

These remarks are not intended as criticism. What Ott demonstrates is the power of the physical approach to mathematics. It allows him to go further into the infinite intricacies of dynamical systems than would be possible using purely mathematical techniques. The chaotic scattering of particles in potential systems becomes something to be observed numerically (experimentally) and carefully unravelled. Just as in mathematical physics, the correspondence between the models and some (ideal?) reality is questionable, so, in this form of physical mathematics, the nature of the conclusions drawn is unclear. Even so, Ott has managed to capture the beauty of this subject in a way that should motivate and inform the next generation of students in applied dynamical systems. □

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Genetic fusion

J. Delharty

Chromosomes: A Synthesis. By Robert P. Wagner, Marjorie P. Maguire and Raymond L. Stallings. Wiley-Liss: 1993. Pp. 523. £75.00, \$89.95.

CHROMOSOMES are currently fashionable mainly because of the rapidly expanding field of molecular cytogenetics and its many applications in human, mammalian and evolutionary genetics. Attention has also focused on the chromosomal assignment of genes as a starting point for their isolation by the Human Gene Mapping Project. So it is undoubtedly a good time to bring out a book that attempts to cover this fusion of classical genetics and molecular biology. *Chromosomes* does this with considerable success.

In a sense, the title is misleading. From the central core of topics related to chromosomes, the book branches out to include almost all of modern genetics. For example, the chapter entitled "Basic chromosome structure" also covers topics such as reassociation kinetics, formation and cloning of DNA molecules, DNA sequencing methods and higher-order organization of chromatin. "Chromosomes and the cell cycle" includes a good mini-review of eukaryotic DNA repair and "Variations in chromatin organization and amount" has a section on transposable elements that includes retroviruses and their relation to human proto-oncogenes.

The book will be a good source of reference and up-to-date information for advanced students of genetics, giving a lead into a wide variety of aspects. In addition to some historical coverage there are many recent examples, principally from *Drosophila*, human and mammalian genetics, with relevant photographic illustrations, although with occasionally less than accurate captions. In general, the figures are clear and informative, but there are lapses into amateurism where the chromosomes appear as squiggles and the labels are microscopically small. More than 50 pages of references are usefully given as a chunk at the end of the book, but organized by chapter for ease of location. The price will be beyond a student budget but librarians and tutors would be well advised to keep copies: it is a volume that will be much sought after. □

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■ Also recently published is *Sex Chromosomes and Sex Determination in Vertebrates* by Alberto J. Solari (CRC Press, \$149.95).

Microwave anisotropies from cosmic defects

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High-resolution maps of the temperature fluctuations in the cosmic microwave background radiation will provide valuable insight into the mechanism of structure formation in the early Universe. Theories involving inflation generally predict a pattern of gaussian (random) noise, whereas theories based on symmetry breaking and the generation of defects—such as strings, textures and monopoles—have more distinctive signatures. Degree-scale measurements of the microwave background currently in progress should provide a powerful test of these competing theories.

THE search for a theory of cosmic structure formation is one of the most exciting areas of science today. Recent observations have drastically tightened constraints on theories, but most basic questions remain open. One of these is whether the primordial perturbations in the Universe took the form of a random-phase superposition of linear waves (that is, gaussian noise), or whether there was important additional structure. The two simplest *ab initio* families of theories are quite distinct in this regard.

The first invokes inflation, a hypothetical epoch of very rapid expansion which made the universe very large and smooth, to stretch microscopic quantum fluctuations to very large length scales. Essentially because the fluctuations must be kept small to preserve the homogeneity of the Universe, the generation mechanism is linear and gaussian fluctuations are predicted.

The second set of theories involves the breaking of symmetries in unified field theories. According to these theories, symmetries are broken as the hot early Universe cooled, forming a disordered phase (like ice forming in rapidly freezing water). This phase would contain defects such as cosmic strings, monopoles or textures¹. As the Universe expands and cools, the tangle of defects straightens itself out on progressively larger scales. This ordering process involves nonlinear physics, which leads to a nongaussian pattern of fluctuations.

Despite the different physics involved, the inflation and defect theories are surprisingly similar in their predictions, and detailed calculations and observations have not yet convincingly ruled either out. Here we argue that this state of affairs should not last long—degree-scale microwave anisotropy data available in the near future could decide the issue.

The cosmic microwave background radiation provides a uniquely clean probe for cosmic defects. As the defects evolve, they produce a time-dependent gravitational field which both disturbs the homogeneity of the Universe and shifts the energy of the cosmic microwave photons passing near the defects. A map of the microwave sky would reveal the pattern of defects in the Universe as seen by their influence on the microwave photons which reach us. For each kind of defect there are specific signatures—cosmic strings would produce linelike discontinuities on the sky², and texture would produce hot and cold spots³. These descriptions are caricatures: in a realistic calculation there are many ways in which the signatures blur or overlap, making the patterns more difficult to tell apart from random gaussian noise. Our work is aimed at quantifying the extent to which realistic defect-induced patterns will ultimately be distinguishable from gaussian noise. We find that one can indeed tell the difference between the texture and monopole theories on the

one hand, and inflationary theories on the other, but a high-confidence result requires the mapping of a large region of the sky with low noise and degree-scale resolution.

Origin of the microwave anisotropy

The simplest defect theories are highly predictive, the perturbations being governed by one parameter, the energy scale ϕ_0 at which the symmetry involved is broken⁴. This is expected to be the energy at which the strong, weak and electromagnetic forces unify, the 'grand unification scale', $\phi_0 \approx 10^{16}$ GeV. The theoretical 'payoff' should one of these theories be verified would be high—maps of the microwave sky would yield information about symmetry breaking at very high energies, specifically the geometry and topology of the vacuum manifold^{1,4}.

The COBE (cosmic background explorer satellite) provided the first evidence of anisotropy on the microwave sky⁵. The amplitude detected cleanly fixes ϕ_0 , the parameter governing the overall magnitude of density perturbations in each theory. Further information is limited by COBE's low resolution—the large (10°) beam blurs any information on smaller scales—and calculations show that its findings are compatible with essentially all the cosmic defect and inflationary theories^{6,7}.

Higher-resolution measurements offer the prospect of definitely excluding theories, and answering the question of gaussianity. The data gathered so far form a partial but nonetheless intriguing picture. The MAX experiment observed two regions of the sky and found one to be quiet and the other noisy—assuming gaussian statistics, discrepant upper and lower 95% confidence bounds were obtained^{8,9}. And the South Pole data of Schuster *et al.*¹⁰ contains a statistically significant nongaussian 'bump'¹¹.

Furthermore, the detected temperature differences $\delta T/T$ vary across a narrow band of angular scales. The PYTHON experiment¹² detects fluctuations at a level $\sim 3 \times 10^{-5}$ on a scale of $\sim 3^\circ$, and MAX at 4×10^{-5} on a scale of $\sim 0.5^\circ$. But the SP91 experiment of Gaier *et al.*¹³ set an upper limit of 1.4×10^{-5} on a scale of $\sim 1^\circ$, and the Schuster *et al.* scan, on deletion of the 'bump', yields a limit of $< 1.0 \times 10^{-5}$ on the same scale¹¹. These differing results do not appear compatible with a gaussian sky with a smooth power spectrum. It is clearly of interest to determine the predictions of the nongaussian theories while the observational situation is still being resolved.

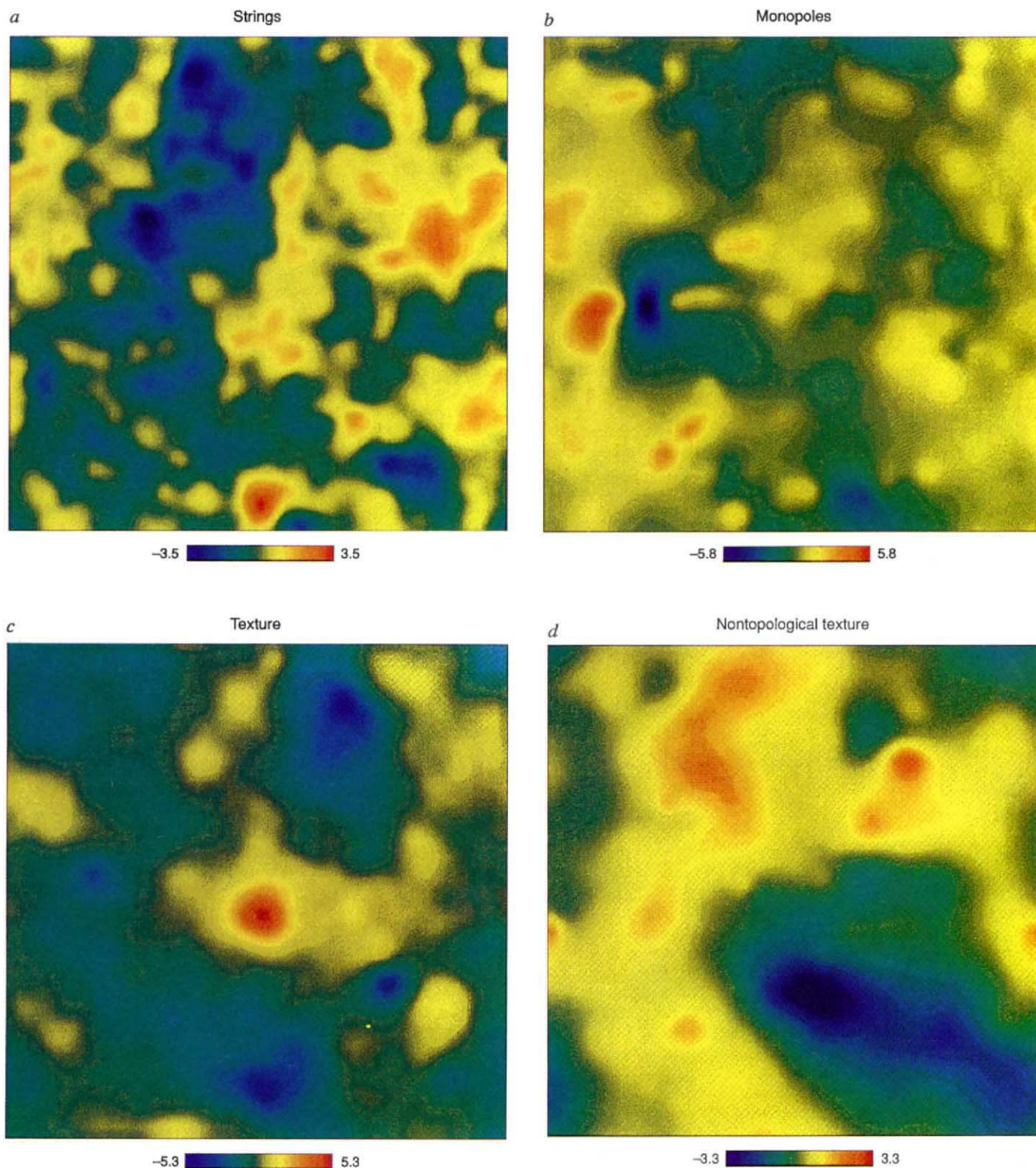
Techniques for realistic calculations of microwave anisotropies in the cosmic defect theories have been recently developed^{6,7}. The idea is to follow the ordering of the defect fields numerically, solve the linearized Einstein equations to determine the gravitational fields produced, and simultaneously calculate the energy

shift for photons on paths converging at an 'observer' at the current epoch. Previously, such calculations have been performed only for comparison with low-angular-resolution experiments such as COBE. As one goes to higher angular resolution, it is important to include the 'Doppler' effect produced by the scattering of photons from free moving electrons. The number of such free electrons depends on the ionization history of the Universe.

According to the standard theory, electrons and protons combined to make neutral hydrogen at a redshift of $\sim 1,000$. But

observations of quasars show that the intergalactic medium was almost fully ionized by a redshift of ~ 5 , probably by the release of ultraviolet radiation from stars. In cosmic defect theories, reionization at redshifts higher than 100 is quite likely⁷, because the defects cause regions of the Universe in their vicinity to undergo gravitational collapse very early. This is quite unlike the case in inflationary scenarios in which it is not clear one can reionize the Universe even by a redshift of ~ 5 (although see refs 14, 15).

The main effect of reionization on the microwave anisotropy



is to smooth it on smaller angular scales. Consider the photons now arriving at us along a given line of sight. If we followed each photon back in time along its path, we would see it follow a random walk, scattering increasingly frequently at higher redshift as the electron density grew. At very early times the photon position would be effectively ‘frozen’ as its mean free path tended to zero. The photons we see along a given direction thus probe the gravitational potential in the early Universe over a length scale λ , the photon mean free path at the time when typical photons ‘last scatter’. Reionization, by increasing photon scattering, smooths the observed pattern of microwave anisotropy. This smoothing is only significant if the reionization occurs early: at redshifts smaller than ~ 50 the density of electrons is so low that scattering has little effect. The angular scale subtended by λ is of the order of 2° without reionization and 6° with reionization. (Throughout this paper we assume the ‘canonical values’ $\Omega = 1$, $\Lambda = 0$, $h = 0.5$, $\Omega_{\text{baryon}} = 0.05$ —but because for many other values the Universe was close to critical density at the relevant epochs, one can simply rescale the angular scale of our maps to obtain the corresponding results.)

If, as we shall assume, early reionization did occur, then the ‘Doppler’ effect must be accurately included in degree-scale calculations. This effect is likely to be more gaussian than the direct gravitational effect of the defects, and an important question is whether the noisy ‘Doppler’ background could mask the distinctive ‘signatures’ of the cosmic defects.

Field and defect evolution

Cosmic defects are inevitable by-products of the symmetry breaking process in unified field theories. They are topological ‘knots’ in a hypothetical symmetry breaking field called the

TABLE 1 Degree-scale temperature gradients

Theory	$\theta \langle \vec{\nabla} \delta T / T ^2 \rangle_{1.5}^{1/2}$	$\theta \langle \vec{\nabla} \delta T / T ^2 \rangle_3^{1/2}$
Strings	$2.4 \pm 0.5 \times 10^{-5}$	$2.4 \pm 0.5 \times 10^{-5}$
Monopoles	$1.6 \pm 0.3 \times 10^{-5}$	$1.9 \pm 0.4 \times 10^{-5}$
Textures	$0.9 \pm 0.2 \times 10^{-5}$	$1.4 \pm 0.3 \times 10^{-5}$
NT textures	$0.9 \pm 0.2 \times 10^{-5}$	$1.4 \pm 0.3 \times 10^{-5}$
Standard CDM	$2.4 \pm 0.5 \times 10^{-5}$	$1.9 \pm 0.4 \times 10^{-5}$
Reionized CDM	$1.4 \pm 0.3 \times 10^{-5}$	$1.7 \pm 0.3 \times 10^{-5}$

Typical values of the temperature gradients, to which many degree-scale experiments are sensitive, in each theory considered here (N.T., nontopological). The sky maps are first smoothed with a gaussian of FWHM θ . The standard deviation of the two-dimensional temperature gradient $\sigma = \langle |\vec{\nabla} \delta T / T|^2 \rangle^{1/2}$ is then calculated. The table shows $\sigma\theta$, the typical temperature gradient on the scale θ , for θ equal to 1.5 and 3 degrees (columns 2 and 3 respectively). All theories have been normalized to fit the standard deviation on the 10° scale reported by COBE, $(\delta T / T)_{10} = 1.24 \pm 0.2 \times 10^{-5}$. This yields the values $G\mu = 2.0 \times 10^{-6}$ for strings, and $8\pi^2 G\phi_0^2 = 6.7 \times 10^{-5}$, 1.1×10^{-4} and 1.8×10^{-4} for monopoles, textures and nontopological textures respectively. Here G is the gravitation constant, μ is the cosmic string tension and ϕ_0 is the symmetry breaking scale. Note that these numbers are for the ‘canonical’ parameters $h = 0.5$, $\Omega = 1$, $\Lambda = 0$, and fractional reionization $f = 1$.

‘Higgs’ field. Depending on the symmetry which is broken, they may take the form of pointlike defects (‘monopoles’), lines (‘strings’), sheets (‘walls’), or a three-dimensional structure known as ‘texture’. For monopoles, texture and nontopological texture, accurate and well tested methods are available to evolve the fields⁷. Strings are more difficult. After nearly a decade of work uncertainties remain¹⁶, stemming from the sensitivity to small-scale structure on the long strings—different ways of handling this numerically lead to different scaling densities for the long strings. Existing codes incorporating cosmic expansion have limited dynamic range (an expansion factor of less than 20). We have attempted instead to model string network evolution by making use of an exact lattice solution to the Nambu equations for string evolution and reconnection first used by Smith and Vilenkin^{17,18}. Mapping coordinates to preserve the causal structure (time to conformal time, space to co-moving coordinate) one obtains a simple model of evolution in an expanding universe. This model exhibits similar scaling densities and ‘fractal’ structure to that of the more complex expanding-universe codes¹⁶, but has the virtue that very large simulations are possible, extending over an expansion factor in excess of 10^4 .

Before this work, Bouchet *et al.*¹⁹ constructed a picture of the anisotropy produced by cosmic strings by stacking expanding-universe simulations end to end, and using a Minkowski space approximation to model the anisotropy pattern. As they emphasized, this exaggerates the linelike discontinuities produced by the strings. We instead use our model string network as a source for the full linearized Einstein equations, which we solve to calculate the photon energy shifts. We also include the effects of electron scattering. Our conclusion is that resolution higher than a degree is needed to see any evidence of the discontinuities from strings.

Photon scattering

The first part of our technique is to construct the photon paths backwards in time, propagating away from the observer. During a time interval $[t_i, t_f]$, the probability for a photon not to scatter is given by

$$P = e^{-d}$$

$$d = \int_{t_i}^{t_f} dt \, cn(t) \sigma_T = cn_o \sigma_T t_o \left[\left(\frac{t_o}{t_i} \right) - \left(\frac{t_o}{t_f} \right) \right] \quad (1)$$

where t is the cosmic time, c is the speed of light, $n(t) \propto t^{-2}$ is the electron density, σ_T is the Thompson cross-section and the

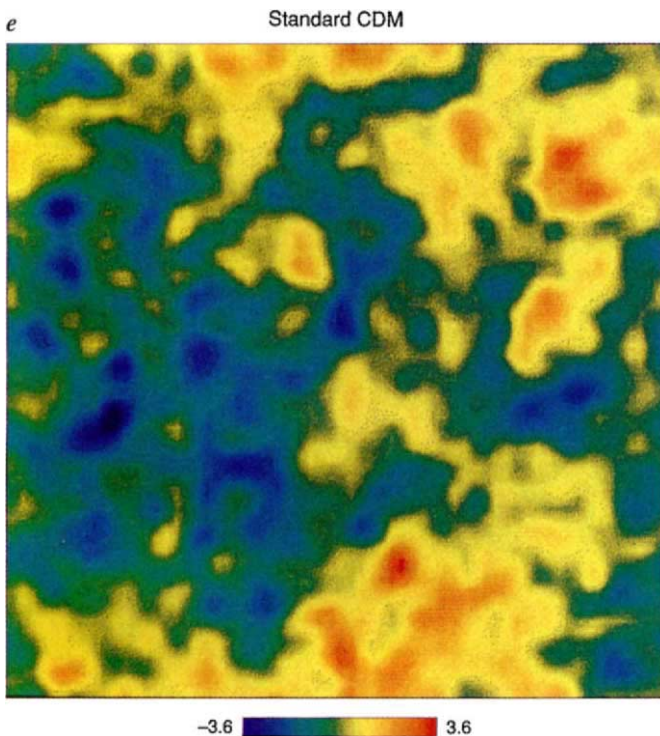


FIG. 1 Temperature anisotropy ($\delta T/T$) maps in the string (a), monopole (b), texture (c), nontopological texture (d) and standard cold dark matter (CDM) (e) scenarios. The side of the box subtends an angular scale of 30° , and $\delta T/T$ is given in units of standard deviation (s.d.) for each map (see colour scale at the bottom of map). All maps have been smoothed with a gaussian of FWHM 1.0° . The nongaussianity is quite apparent in the texture and monopole maps (note the 5 s.d. peaks and troughs), much less so in the string and nontopological texture theories.

subscript o refers to the present²⁰. We apply equation (1) at each time step of the photon path, drawing a random number to determine whether a scattering actually occurred. If it did, a new direction is chosen with a probability given by the Thompson differential cross-section, and the scheme iterates. When the photon mean free path falls below our resolution scale, we store the entire set of photon paths, and begin the main calculation.

The main calculation evolves photons, defect fields and gravitational field variables forwards in time from $t=0$ to the present epoch. The defect fields start out uncorrelated, taking random values at each grid point. Using the stress energy from the defect fields, we solve the linearized Einstein equations using the analytical formulae of ref. 7, obtaining the perturbation to the spacetime metric $h_{ij}(\eta, \mathbf{x})$. This is then used to calculate the energy shift the photons experience, through the 'Sachs-Wolfe' formula

$$\frac{\delta T}{T}(\mathbf{n}) = -\frac{1}{2} \int_0^{\eta} d\eta h_{ij,0}(\eta, \mathbf{x}(\eta)) n^i n^j \quad (2)$$

Here $\mathbf{n}$ is the direction of the line of sight, $\mathbf{x}(\eta)$ is the photon path, η is a time coordinate known as 'conformal' time, and $h_{ij,0}$ is the partial derivative of $h_{ij}(\eta, \mathbf{x})$ with respect to η . This formula relates the shift in the energy of a photon to the change in its proper length as it travels to us.

We include in our calculation the 'Doppler' effect mentioned above in the approximation that at the epochs of interest the electron velocity is equal to the velocity of the dark matter. This is reasonable because photon drag is small at the redshifts of interest²¹.

A typical run employs a box of 128^3 for the field evolution (320^3 for strings), 64^3 for the metric perturbations and photon trajectories, with 100 photons along each of 64^2 lines of sight. The set of photon paths are set up as described above by evolving the photons backwards in time along lines emanating from an 'observer', where they subtend a square 30° on a side. Then the whole simulation, including photons, defect fields and spacetime metric variables $h_{ij,0}$ is evolved forwards from $t=0$ to the present.

Six sets of photons are used, each set converging to an 'observer' at the centre of one of the faces of the simulation volume. The code accurately reproduces known analytical solutions for anisotropies caused by straight strings, oscillating loops and collapsing textures. We have performed exhaustive tests for finite size and resolution effects, which will be reported elsewhere (D.C., P.F., P.G. and N.T., manuscript in preparation). Finite grid effects are present, but become small on angular scales corresponding to a few grid spacings and above. We smooth our final maps on a 1° scale (four field grid spacings, eight string grid spacings) and believe they are trustworthy on this scale.

Results

Sky maps of the cosmic microwave background anisotropy produced by cosmic defects are shown in Fig. 1a–d. Figure 1e shows an equivalent map for the simplest inflationary model, commonly referred to as standard cold dark matter (CDM) theory (see for example ref. 22). This theory provides us with a useful gaussian benchmark with which to compare the nongaussian defect theories. All maps are 30° on a side and have been smoothed with a gaussian window of full-width half-maximum (FWHM) 1° .

In Fig. 2, we show the power spectra of spherical harmonics on the sky, calculated by averaging over 18 maps obtained from 3 independent simulations for each theory. For comparison the power spectra for standard CDM²², with and without early reionization are also shown. All theories are normalized to fit the COBE results (as corrected in ref. 23) on large angular scales, assuming $\Omega=1$, $\Lambda=0$. At small Legendre coefficient l the defect theory results are consistent with previous all-sky simulations⁷. The error bars show the 'cosmic variance'—clearly many such patches on the sky will be necessary to obtain a good estimate of the power spectrum at small l .

There is a striking suppression of power at higher l in the defect theories, most significantly in the texture and nontopological texture theories. This is due to reionization—in the absence of reionization we would expect an approximately 'scale-

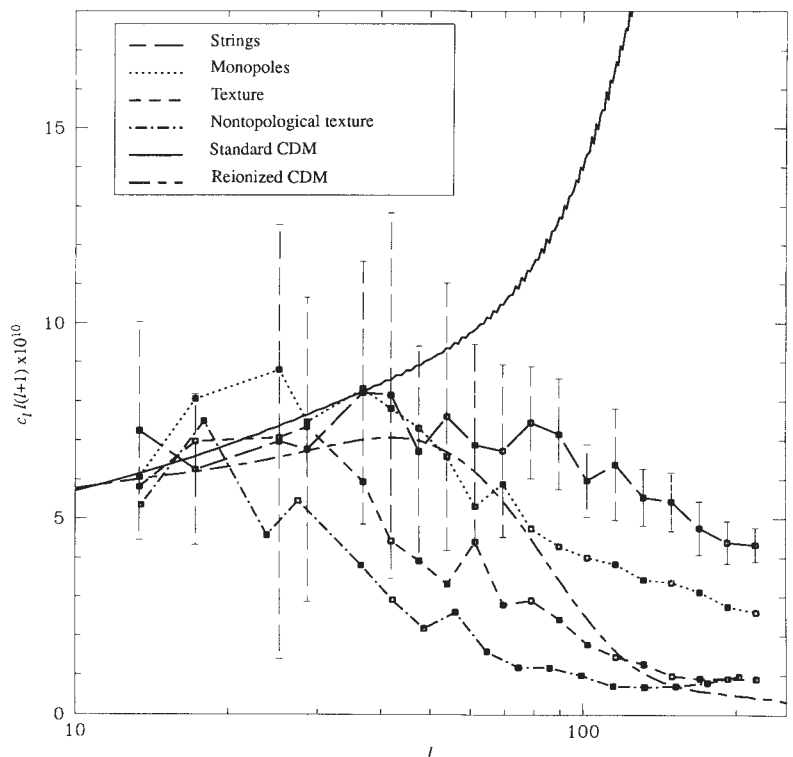


FIG. 2 Anisotropy power spectra for the cosmic defect theories. The temperature pattern on each map (Fig. 1a–e) is expanded as a sum of spherical harmonics $Y_{lm}(\theta, \phi)$, $(\delta T/T)(\theta, \phi) = \sum a_{lm} Y_{lm}(\theta, \phi)$, with θ, ϕ the usual polar angles. The power spectrum c_l is defined as the ensemble average $\langle |a_{lm}|^2 \rangle$. The power per logarithmic interval in l is then $l^2 c_l$, constant for a scale-invariant pattern. All theories considered here are approximately scale invariant for harmonics with $l < 10$, to which COBE is sensitive. Differences appear at higher l . For the defect theories, the curves show the average power spectrum for 18 maps. The error bars on the strings curve show the standard deviation (the 'cosmic variance'). The standard deviation is similar in the other theories. The solid curve shows the spectrum for the 'standard CDM' model, and the dashed line that for the same theory with early reionization assumed.

TABLE 2 Nongaussianity in cosmic defect anisotropy maps

Theory	0°	1.2°	2.4°	3.6°
Strings	4	2	2	0
Monopoles	18	18	18	13
Textures	18	17	6	2
N.T. textures	4	1	1	1
Standard CDM	0	0	0	0

The number of maps, out of 18 constructed for each theory, that were 'significantly nongaussian', defined as follows. Maps passing the test were those which (after smoothing with a gaussian of FWHM 1.2°, 2.4° or 3.6°) had a larger maximum temperature gradient than each of 99% of the gaussian ensemble of 1,000 maps based upon it (see text). A similar procedure could in principle be followed with observational data.

invariant' (flat) power spectrum at higher l . It should be noted that in the defect theories the 'Doppler' effect mentioned above is relatively small. This effect causes the dramatic rise in the inflationary spectrum visible in Fig. 2. The reason for this difference is that if both inflationary and defect theories are normalized to COBE (at low l), the r.m.s. velocities induced in the defect theories are relatively small compared to those in inflation. Inflationary fluctuations are 'built in' whereas in the defect theories they are 'induced' by motion of the defect fields, requiring larger gravitational fields for a given velocity⁷.

In Table 1 we show a quantity more closely related to what experiments measure, the r.m.s. temperature gradients in each theory. We caution that these are strongly dependent on the values of as-yet poorly determined cosmological parameters, h , Λ and Ω . In an open universe, or in a Λ -dominated flat universe, there would be some suppression of the anisotropy on COBE scales, with a relative increase in the power on degree scales. Likewise, if reionization were less than 100% efficient, there would be more power on smaller scales.

Nongaussianity

The main focus of our work has been to determine whether the specific patterns expected in the defect theories make them readily distinguishable from gaussian scenarios. The maps provide visible indications of this—the texture and monopole maps have peaks and troughs in excess of 5 standard deviations (s.d.), with a characteristic structure. For textures some maps like Fig. 1c show clear, isolated hot and cold spots a few degrees across as expected³. For monopoles, all of our maps show many pairs of hot regions separated by a colder region, a pattern one expects from an annihilating monopole–antimonopole pair. Most of these are small, but a few are still visible even after degree-scale smoothing. Figure 1b shows one large monopole–antimonopole pair annihilating to the left and right of the cold spot left of centre of the picture, and one smaller diagonal pair just below that. The string maps do show evidence of lines along which the temperature changes sharply from hot to cold, but they are not overwhelming different from standard CDM.

Objective statistical criteria are needed to determine whether

a given sky map is nongaussian or not. As our basic method, we take each sky map and randomize the phases of the Fourier components, thus constructing an ensemble of 1,000 gaussian maps with the same power spectrum. We then search for statistics which strongly distinguish the original maps from the gaussian ensemble. We found measures based on the distributions of the temperature anisotropy δT itself to be rather weak. The simplest are the skewness $\langle \delta T^3 \rangle / \langle \delta T^2 \rangle^{3/2}$ and kurtosis $(\langle \delta T^4 \rangle - 3\langle \delta T^2 \rangle^2) / \langle \delta T^2 \rangle^2$, both zero for a gaussian distribution. Using 18 maps for each theory, we calculated the mean skewness and kurtosis of the temperature anisotropy δT . None were statistically significant. Likewise the maximum temperature was not a very powerful discriminator. Some maps do have 5 s.d. peaks, like those shown in Fig. 1, but a significant number of the maps do not.

In previous work¹¹, we suggested that large gradients might be a better way to distinguish the nongaussian from gaussian theories, and we have found this to be a powerful idea in the present study. The defects are localized regions of high energy density: their motion is relativistic, and produces large, rapid changes in the energy density. The resulting gravitational field causes large shifts in the energy of photons passing through—causing sharp gradients to be seen in the temperature pattern on the microwave sky. One simple test is as follows. A map is considered 'significantly nongaussian' if the maximum gradient in it is larger than that in 99% of the ensemble of gaussian maps based upon it. Table 2 shows the number (out of 18) of the maps satisfying this criterion for each theory. Most of the texture and monopole maps were strongly nongaussian by this criterion, the string maps much less so.

One would prefer a statistic less sensitive to extreme data points, but the significance of the nongaussianity increases with increasing resolution, and it is the small-scale structure of the relatively rare defect-induced signatures that is clearly the most significant feature. This suggests that the optimal experimental strategy to establish nongaussianity would be to measure temperature gradients at lower (1°) resolution, and then 'focus in' on regions of high gradient with higher resolution.

For strings, the evidence for nongaussianity is weak. The sharp discontinuities produced by strings are not readily apparent on a 1° smoothing scale. On smaller scales, a realistic calculation would require techniques which include the effects of Doppler scattering more accurately.

Conclusions

Our conclusion is that the goal of distinguishing between the texture and monopole theories from gaussian theories based on inflation should be achievable from microwave anisotropy data available soon. One difference is in the spectrum of spherical harmonics, due both to reionization and the difference between 'induced' and 'built-in' cosmological perturbations. This is very dependent on as-yet poorly determined cosmological parameters. More distinctive is the nongaussianity of the anisotropy pattern which may even be detectable by degree-scale experiments currently in progress. □

Received 20 October 1993; accepted 2 February 1994.

- Kibble, T. W. B. *J. Phys.* **A9**, 1387–1398 (1976).
- Kaiser, N. & Stebbins, A. *Nature* **310**, 391–393 (1984).
- Turok, N. & Spergel, D. *Phys. Rev. Lett.* **64**, 2736–2739 (1990).
- Turok, N. *Physica Scripta* **T36**, 135–141 (1991).
- Smoot, G. F. et al. *Astrophys. J.* **396**, L1–L5 (1992).
- Bennett, D. P. & Rhee, S. H. *Astrophys. J.* **406**, L7–L10 (1993).
- Pen, U., Spergel, D. & Turok, N. *Phys. Rev. D* **49**, 692–729 (1994).
- Gundersen, J. O. et al. *Astrophys. J.* **413**, L1–L5 (1993).
- Meinhold, P. et al. *Astrophys. J.* **409**, L1–L4 (1993).
- Schuster, J. et al. *Astrophys. J.* **412**, L47–L50 (1993).
- Graham, P., Turok, N., Lubin, P. M. & Schuster, J. *Astrophys. J.* (in the press).
- Dragovan, M. et al. *Astrophys. J.* (in the press).
- Gaier, T. et al. *Astrophys. J.* **398**, L1–L4 (1992).
- Silk, J. & Tegmark, M. *Astrophys. J.* (in the press).
- Fukugita, M. & Kawasaki, M. Tokyo Univ. Report No. 301-93-13 (1993).
- Gibbons, G., Hawking, S. & Vachaspati, T. (eds) *The Formation and Evolution of Cosmic Strings* (Cambridge Univ. Press, 1990).

- Smith, G. & Vilenkin, A. *Phys. Rev. D* **36**, 990–996 (1987).
- Sakellariadou, M. & Vilenkin, A. *Phys. Rev. D* **42**, 349–353 (1990).
- Bouchet, F., Bennett, D. P. & Stebbins, A. *Nature* **335**, 410–414 (1988).
- Peebles, P. J. E. *Astrophys. J.* **315**, L73–L75 (1987).
- Peebles, P. J. E. *Principles of Physical Cosmology* (Princeton Univ. Press, 1993).
- Crittenden, R., Bond, J. R., Davis, R. L., Efstathiou, G. P. & Steinhardt, P. J. *Phys. Rev. Lett.* **71**, 324–327 (1993).
- Wright, E. et al. COBE preprint No. 93-06 (Goddard Space Flight Center, Greenbelt, 1993).

ACKNOWLEDGEMENTS. We thank U. Pen for collaboration in the early stages of this project, and particularly for his help with computational aspects. We thank R. Crittenden, R. Davis and P. Steinhardt for allowing us to use their standard CDM power spectra for the comparisons made in Fig. 1 and 2. D.C. thanks the UK SERC for financial support, while P.F. thanks Programa Ciencia (Portugal). The work of N.T. was partially supported by the UK SERC, the US NSF and the David and Lucile Packard Foundation.

2.2 Mb of contiguous nucleotide sequence from chromosome III of *C. elegans*

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As part of our effort to sequence the 100-megabase (Mb) genome of the nematode *Caenorhabditis elegans*, we have completed the nucleotide sequence of a contiguous 2,181,032 base pairs in the central gene cluster of chromosome III. Analysis of the finished sequence has indicated an average density of about one gene per five kilobases; comparison with the public sequence databases reveals similarities to previously known genes for about one gene in three. In addition, the genomic sequence contains several intriguing features, including putative gene duplications and a variety of other repeats with potential evolutionary implications.

THE free-living nematode *Caenorhabditis elegans* is an excellent model organism for the study of development and behaviour, and its small size and short life cycle greatly facilitate genetic analysis^{1,2}. Because a nearly complete clonal physical reconstruction of the genome is available³⁻⁵, and 1,200 genetic loci have been identified, the nematode is also a good candidate for complete DNA sequence analysis. The haploid genome of *C. elegans* contains approximately 100 Mb (megabases) distributed on six chromosomes². Many of the genes required for normal development and behaviour in the nematode have extensive similarity to their mammalian counterparts. However, compared with mammalian genes, genes in *C. elegans* typically have smaller and fewer introns^{2,6}, thus simplifying the identification of previously uncharacterized genes. Many of these newly identified genes may in turn be used to probe for as-yet unidentified mammalian genes.

As previously reported⁶, we have embarked on a collaborative project to sequence the entire genome of *C. elegans*. Here we report some of the results from the first three years of the pilot phase, a point at which each laboratory has completed one contiguous megabase of genomic sequence. The combined data represent a sequence spanning more than 2.1 Mb. Most of the sequence derives from cosmid clones that were mapped to chromosome III by restriction fingerprinting³. At this physical map location, there are two large cosmid contigs, each more than 1 Mb long and bridged by a yeast artificial chromosome (YAC) clone. A small cosmid contig of 92 kilobases (kb) lies near the centre of the YAC bridge. Our genomic sequence analysis of this large region of chromosome III has confirmed the high gene density that we found in the first three sequenced cosmids⁶, and has resulted in the identification of many more genes and other interesting sequence features.

Sequencing strategy

At the start of the pilot phase, we experimented with a primer-directed or 'walking' strategy in which site-specific oligonucleotide primers were used to extend sequences sequentially from a limited number of starting points⁷. This was initially done using cosmid DNA as template for the sequencing reactions. However, because cosmid DNA proved difficult to purify in sufficient

quantities and was troublesome because of the presence of repeated sequences, we used random phagemid and M13 subclones as templates for the walking strategy^{8,9}. During this work, we found that a combination of small insert (1–2 kb) and large insert (6–9 kb) subclones provided representative coverage for a typical cosmid. Even with subclones, primer-directed sequencing was occasionally problematic because of the presence of repeated sequences. Thus, our strategy has evolved to the point where most sequence data come from the initial readings of 600–800 random subclones. As the use of automated fluorescent DNA sequencing machines for data collection provides high-throughput sample processing^{10,11}, this approach is efficient and cost-effective. This random or 'shotgun' phase not only provides much of the final sequence, but also maps the subclones needed for closing gaps and completing the complementary strand. At this point, a walking strategy can be successfully exploited for completion, as most repeated sequences in the cosmid insert can be identified and selectively avoided. Many of the details of our sequencing strategy have been reported elsewhere^{6,12–23}.

Sequences were considered to be finished when every base had been determined on both strands and all ambiguities had been resolved. At this point, the finished sequence was compared with the public sequence databases using the BLASTX program for protein similarities and BLASTN for nucleotide similarities²⁴. When similarity searches were complete, likely genes were identified using GENEFINDER (P.G. and L.H., unpublished), and in some cases interactively edited using ACEDB (R.D. and J.T.-M., unpublished). Finished sequences were annotated with regard to likely genes, homologies, *trans*-spliced leaders²⁶, complementary DNA matches^{27,28} and other features, such as structural RNA genes and transposons, and submitted to the GenBank and EMBL databases. Also, to present genomic sequence data in the context of the physical and genetic maps of the nematode, all sequences are deposited in the *C. elegans* database ACEDB, which is available to the research community.

Sequences

Table 1 gives the cosmid clones that were sequenced, along with their database accession numbers and lengths. Most of the redundant overlapping data have been omitted from the data-

TABLE 1 Sequences submitted to the GenBank and EMBL databases

Cosmid name	Locus name	Acc. no.	Length (bp)	Cosmid name	Locus name	Acc. no.	Length (bp)
ZK112	CELZK112	L14324	38,269	K02D10	CELK02D10	L14710	18,683
ZC97	CELZC97	L14714	4,166	F54F2	CELF54F2	L23645	39,573
ZK686	CELZK686	L17337	11,435	F44E2	CELF44E2	L23646	33,651
C08C3	CELC08C3	L15201	44,025	pAR3	CELPAR3	U00026	4,590
C27D11	CELC27D11	L23650	9,973	K01F9	CEK01F9	Z22175	19,834
ZK652	CELZK652	L14429	36,052	ZK637	CE1	Z11115	40,699
C02C2	CELC02C2	L23649	20,495	ZK638	CEZK638	Z12018	1,762
ZK688	CELZK688	L16621	36,977	ZK643	CEZK643	Z11126	39,534
C29E4	CELC29E4	L23651	40,050	R08D7	CER08D7	Z12017	27,368
F54H12	CELF54H12	L25599	19,168	F59B2	CEF59B2	Z11505	43,782
C06G4	CELC06G4	L25598	29,122	R107	CER107	Z14092	40,970
F44B9	CELF44B9	L23648	36,327	F02A9	CEF02A9	Z19555	26,242
K12H4	CELK12H4	L14331	38,582	ZK507	CEZK507	Z29116	13,501
K06H7	CELK06H7	L15314	22,073	ZK512	CEZK512	Z22177	36,997
C14B9	CELC14B9	L15188	43,492	F54G8	CEF54G8	Z19155	31,613
D2007	CELD2007	L16560	13,651	ZC84	CEZC84	Z19157	38,955
C50C3	CELC50C3	L14433	44,733	T23G5	CET23G5	Z19158	26,926
C30A5	CELC30A5	L10990	27,743	T02C1	CET02C1	Z19156	10,308
C02F5	CELC02F5	L14745	22,333	M01A8	CEM01A8	Z27081	19,001
F09G8	CELF09G8	L11247	41,449	K01B6	CEK01B6	Z22174	34,002
F10E9	CELF10E9	L10986	32,733	C40H1	CEC40H1	Z19154	27,271
ZC262	CELZC262	L23647	4,166	K04H4	CEK04H4	Z27078	33,930
R05D3	CELR05D3	L07144	38,810	C38C10	CEC38C10	Z19153	34,193
ZK353	CELZK353	L15313	24,916	T26G10	CET26G10	Z29115	30,251
ZK1236	CELZK1236	L13200	28,878	F54C8	CEF54C8	Z22178	23,000
C30C11	CELC30C11	L09634	30,865	B0464	CEB0464	Z19152	40,090
F42H10	CELF42H10	L08403	28,687	F55H2	CEF55H2	Z27080	22,950
C04D8	CELC04D8	L16687	10,433	ZK1098	CEZK1098	Z22176	37,310
ZC21	CELZC21	L16685	36,087	C48B4	CEC48B4	Z29117	35,000
K10C7 (C02D5)	CELC02D5	L16622	28,735	F58A4	CEF58A4	Z22179	38,000
C06E1/F43A9	CELC06E1	L16560	40,216	C15H7	CEC15H7	Z22173	28,000
C13G5	CELC13G5	L14730	10,883	C07A9/C40D10	CEC07A9	Z29094	66,004
F22B7	CELF22B7	L12018	40,222	T05G5	CET05G5	Z27079	36,180
B0523	CELB0523	L07143	14,334	R10E11	CER10E11	Z29095	32,254
B0303	CELB0303	M77697	41,071	ZK632	CEZK632	Z22181	36,000
ZK370	CELZK370	M98552	37,675	K11H3	CEK11H3	Z22180	33,000
pAR2	CELPAR2	U00025	12,721	ZK757	CEZK757	Z29121	31,000

base entries, although neighbouring sequences typically contain a small number of overlapping bases to facilitate joining or to keep a gene intact. The total non-overlapping sequence assembled from all of the clones in Table 1 spans 2.181 Mb. A map of this region showing the sites of predicted genes and previously known loci is presented in Fig. 1. Several additional cosmids which extend the sequence more than 2,000 kb to the left and 500 kb to the right are in various stages of completion.

The most striking result from genomic DNA sequencing is the continued high number of predicted genes in the region. In the 2.181 Mb of genomic sequence reported here, 483 putative genes were identified by similarity searches and GENEFINDER analysis. Start points for these candidate genes are indicated for both strands of the genomic sequence in Fig. 1. Generally, genes seem to be dispersed evenly throughout the region, with only a few examples of apparent clustering. One of the most gene-dense regions is contained in cosmids C30A5, C02F5 and F09G8 (Fig. 1, 492–553 kb). Here a 61-kb region contains 141 exons (in 24 putative genes), which account for 47% of the sequence. When the introns are also considered, more than 80% of the bases in this region are within predicted genes. Also in a 69.5-kb stretch of genomic DNA (F55H2, ZK1098; Fig. 1, 1,770–1,840 kb), 109 exons (in 19 putative genes) are predicted, with 40% of this region representing coding sequence and a total of 65% contained in genes. The longest stretch of genomic sequence that does not contain a likely gene is ~25 kb (pAR3, K01F9; Fig. 1, 1,145–1,170 kb). By comparison, the entire 2.181-Mb region is 29% coding sequence, with a total of 48% representing putative exons and introns. In our analysis of the first 0.1% of the genome, genes were found every 3–4 kb on average⁶. With more than 2% of the genomic DNA sequence now completed, the density is one gene every 4.5 kb. Because the region is expected to be relatively gene-rich², we cannot use this density to extrapolate the total gene number. However, an estimate independent of gene density can be made using the number of tagged cDNAs

which hit candidate genes in the sequence²⁷. Because 125 of the 4,615 *C. elegans* cDNA tags match predicted genes in the sequence, we can now estimate that the genome contains about 17,800 genes ($483 \times 4615/125$) for an average density of one gene every 5.6 kb.

There are no clear examples of genes that overlap, either on the same or on complementary strands. Several cases of orphan open reading frames that have high GENEFINDER scores and that overlap or are contained within candidate genes were observed, although there are no data to indicate that any of these is expressed. When the translated sequence is used in all six reading frames to search the public databases using the program BLAST, approximately one-third of the genes find significant matches to proteins from organisms other than *C. elegans*, with several very highly conserved genes indicated (Table 2). As can be seen in Table 2, a wide variety of different functions are represented, and with the exception of a homeobox cluster (see below), there is no obvious clustering of genes with related function. Most of the matches represent cross-phylum matches, for very little sequence from other nematodes is present in the databases. The fraction of genes with cross-phylum matches will increase as more genes from other organisms are entered into the database, but our previous analysis of these ancient conserved regions indicates that it is unlikely to rise above 40%, as most ancient conserved regions are already represented in the databases²⁹. Although the data are inconclusive, preliminary evidence suggests that the fraction of matches to vertebrate proteins will be similar to this for any non-vertebrate genome.

Known genes

Several interesting genes had been positioned in this region before our sequence analysis. The first 120 kb of the sequence reported here is part of the HOM homeobox gene complex which extends an additional 150 kb to the left^{30–33}. The region is centred around the *Antennapedia*-like gene *mab-5*, which is responsible

TABLE 2 Gene candidates showing significant similarity to existing protein sequences

Gene name	Closest DB hit, Acc. no.	Closest block	Description
ZK112.2	TVMSBF, A40951	—	Kinase-related transforming protein
ZK112.7	A41087	BL00232	Tumour suppressor
ZK686.2	DB73, DROME, P26802	BL00039	ATP-dependent RNA helicase
ZK686.8	A24148	—	N-acetyllactosamine synthetase
C08C3.1	HM11, CAEEL, P17486	BL00027	<i>C. elegans</i> Hox protein egl-5 (ceh-11) gene
C08C3.3	HMMA, CAEEL, P10038	BL00027	<i>C. elegans</i> Hox protein mab-5
ZK652.4	R5RT35, A34571	BL00579	60S ribosomal protein L35
ZK652.5	A34218	BL00027	Distal-less homeotic protein
ZK652.6	S29962	—	ref(2)P protein, Zn-finger region
ZK652.8	C35815	—	Myosin heavy chain-3
C02C2.1	TYRO, STRGA, P06845	BL00497	Tyrosinase
C02C2.3	ACHG, RAT, P18916	BL00236	Acetylcholine receptor
C02C2.4	S27951	—	Sodium/phosphate transport protein
ZK688.8	A24148	—	N-acetyllactosamine synthase
C29E4.3	RNA1, YEAST, P11745	—	RNA production/processing
C29E4.7	S16267	—	Auxin-induced protein
C29E4.8	JS0422, JS0422	BL00113	Adenylate kinase
F54H12.1	ACON, YEAST, P19414	BL00450	Aconitate hydratase
F54H12.6	EF1B, BOMMO, P29522	—	Elongation factor 1 β
C06G4.2	CAP3, RAT, P16259	—	Calpain
C06G4.5	SSR3, MOUSE, P30935	BL00237	Somatostatin receptor
F44B9.1	ACPH, RAT, P13676	BL00708	Acylamino-acid-releasing enzyme
F44B9.8	A45253	—	Replication factor C
F44B9.9	PARB12, S11060	BL00125	Protein phosphatase
K12H4.1	JQ1397	—	<i>Drosophila melanogaster</i> Prospero
K12H4.4	SPC2, CHICK, P28687	—	Signal peptidase
K12H4.8	DEAD, ECOLI, P23304	BL00039	ATP-dependent RNA helicase dead
K06H7.1	S22127	BL00107	Protein kinase
K06H7.3	IPPI, YEAST, P15496	—	Isopentenyl-diphosphate δ -isomerase
K06H7.4	S24168	—	Sec7
K06H7.8	YCK1, YEAST, P23291	—	Casein kinase I
C14B9.1	CRAB, HUMAN, P02511	—	α -B-crystallin
C14B9.2	ER72, MOUSE, P08003	BL00194	Deoxycytidine kinase
C14B9.4	S22127, S22127	BL00098	Protein kinase
C14B9.7	R5RT21, A33295	—	Ribosomal protein L21
C14B9.8	S24109	—	Phosphorylase kinase
D2007.5	KERB, AVIER, P00535	—	ERB-B tyrosine kinase
C50C3.3	SPCN, CHICK, P07751	BL00545	Spectrin α -chain
C50C3.5	TPC1, BALNU, P21797	BL00018	Calmodulin
C50C3.7	OCR1, HUMAN, Q01986	—	Inositol polyphosphate-5-phosphatase
C50C3.11	JH0565	—	Calcium channel α -2b chain
C30A5.1	GRR1, YEAST, P24814	—	GRR1 protein (same as C02F5.7)
C30A5.3	S30854	BL00125	Phosphoprotein phosphatase
C30A5.4	SYB, DROME, P18489	BL00417	Synaptobrevin
C30A5.6	UN86, CAEEL, P13528	BL00035	Unc-86 alternate protein
C30A5.7	UN86, CAEEL, P13528	BL00035	Unc-86 protein
C02F5.3	JC1349	—	GTP-binding protein
C02F5.7	GRR1, YEAST, P24814	—	Glucose-induced repressor (GRR1)
C02F5.9	PRC5, HUMAN, P20618	BL00631	Proteasome component C5
F09G8.3	RS9, BACST, P07842	BL00360	Ribosomal protein S9
F09G8.6	A37122	—	Cuticle collagen
ZC262.3	A43425	—	N-CAM Ig domain
ZC262.5	ATPE, BOVIN, P05632	—	ATP synthase ϵ -chain
R05D3.1	TOPB, HUMAN, Q02880	BL00177	DNA topoisomerase II homologue
R05D3.3	ZG44, XENLA, P18721	—	Gastrula zinc-finger protein
R05D3.6	ATPE, BOVIN, P05632	—	ATP synthase ϵ -chain
R05D3.7	KINH, LOLPE, P21613	BL00411	Kinesin heavy chain
ZK353.6	AMPA, RICPR, P27888	BL00631	Aminopeptidase
ZK1236.1	LEPA, ECOLI, P07682	BL00301	LepA
ZK1236.2	NUCL, RAT, P13383	—	Nucleolin
C30C11.2	DXA2, MOUSE, P14685	—	Diphenol oxidase
C30C11.4	S30788	BL00297	HSP Msi3p
F42H10.4	GYRTI, A03270	—	Cysteine-rich intestinal protein
C04D8.1	SPCB, DROME, Q00963	—	Spectrin β -chain
ZC21.2	JH0588	—	Trp protein
ZC21.3	S14548	—	Dual bar protein
ZC21.4	S29956	—	Breakpoint cluster region (Bcr) protein
C02D5.1	ACDL, RAT, P15650	BL00072	Acyl-CoA dehydrogenase
C06E1.10	S22609, S22609	BL00690	Hypothetical protein
C06E1.4	B40171	—	Glutamate receptor
C06E1.8	B60191	—	Zn-finger
C06E1.9	A31922	—	ATP-dependent RNA helicase
C13G5.1	F34510	BL00027	Engrailed homeotic protein
F22B7.4	S18345	—	Environmental stress protein
F22B7.5	DNAJ, ECOLI, P08622	BL00636	DnaJ
F22B7.6	MUCB, ECOLI, P07375	—	Mucb protein
F22B7.7	S09048	—	Potassium channel protein Hak-6
B0523.1	S00904	BL00239	Tyrosine kinase
B0523.5	GELS, MOUSE, P13020	—	Gelsolin (flightless-1)
B0303.1	CYYA, YEAST, P08678	—	Adenylate cyclase
B0303.2	PNMT, BOVIN, P10938	—	Phenylethanolamine-N-methyltransferase
B0303.3	THIL, RAT, P17764	BL00098	Acetyl-CoA acetyltransferase
B0303.5	YT31, CAEEL, P03934	—	Tc3
B0303.7	NCF2, HUMAN, P19878	—	SH3 domain
B0303.9	SLP1, YEAST, P20795	—	SLP-1
ZK370.3	TALI, MOUSE, P26039	—	Talin
ZK370.4	KAPR, DICDI, P05987	—	Cyclic AMP-dependent protein kinase
ZK370.5	BCKD, RAT, Q00972	—	3-Methyl-2-oxobutanoate dehydrogenase
K02D10.1	S16088, S16088	—	4-Nitrophenylphosphatase
K02D10.5	S07258, S07258	—	<i>Escherichia coli</i> plasmid RK2 gene for P116
F54F2.1	ITAP, DROME, P12080	BL00242	Vitronectin receptor α -subunit

TABLE 2—Continued

Gene name	Closest DB hit, Acc. no.	Closest block	Description
F54F2.2	A44067	—	109K basic protein H
F44E2.1	S08405	—	Protease
F44E2.3	S28589	—	DnaJ
F44E2.4	S03430	—	LDL receptor
F44E2.6	PILB_NEIGO, P14930	—	PILB protein
F44E2.7	CALD_CHICK, P12957	—	Caldesmon
ZK637.1	STP1_ARATH, P23586	—	Sugar transporter
ZK637.10	GSHR_HUMAN, P00390	BL00076	Glutathione reductase
ZK637.11	CC25_SCHPO, P06652	—	CDC25
ZK637.13	GLBH_TRICO, P27613	—	Globin
ZK637.14	PICO_HSV11, P08393	BL00518	Transactivator ICPO (motif 1)
ZK637.5	ARSA_ECOLI, P08690	—	ArsA
ZK637.8	VPP1_RAT, P25286	—	Proton pump
ZK643.2	DCTD_BPT2, P00814	—	DCMP deaminase
ZK643.3	CALR_PIG, P25117	BL00649	G-protein-coupled receptor
R08D7.5	CATR_CHLRE, P05434	BL00018	Calcium-binding protein
R08D7.6	CNAG_BOVIN, P14099	BL00126	Cyclic GMP phosphodiesterase
F59B2.3	NAGA_ECOLI, P15300	—	N-acetyl-glucosamine-6-phosphate deacetylase
F59B2.7	RAB6_HUMAN, P20340	—	Rab6 (Ras protein)
R107.7	GTP_CAEEL, P10299	—	Glutathione S-transferase P subunit
LIN12A.cds	LI12_CAEEL, P14585	BL00022	Lin-12/Notch, EGF and ankyrin repeats
F02A9.5	PCCB_RAT, P07633	—	Propionyl-CoA carboxylase
GLP1A.cds	GLP1_CAEEL, P13508	BL00022	Lin-12/Notch, EGF and ankyrin repeats
ZK507.1	HR25_YEAST, P29295	BL00107	HRR25 protein kinase
ZK507.6	CG2A_PATVU, P24861	BL00292	G2/M cyclin A
ZK512.2	SPB4_YEAST, P25808	BL00039	RNA helicase
ZK512.4	SRP9_CANFA, P21262	—	Signal recognition particle 9K protein
F54G8.2	KDGL_PIG, P20192	BL00479	Diacylglycerol kinase
F54G8.3	ITA3_CRISP, P17852	BL00242	Integrin α -chain
F54G8.4	KRET_HUMAN, P07949	BL00518	Ret zinc-finger region
ZC84.1	LACI_RABIT, P19761	BL00280	Serine protease inhibitor
ZC84.2	CNGC_RAT, Q00195	—	Cyclic nucleotide gated olfactory channel
ZC84.4	GASR_RAT, P30553	BL00237	G-protein-coupled receptor
T23G5.1	RIR1_HUMAN, P23921	BL0089	Ribonucleoside-diphosphate reductase lg chain
T23G5.2	SC14_KLULA, P24859	—	SEC14 (yeast)
T23G5.5	NTTN_HUMAN, P23975	BL00610	Neurotransmitter transporter
T02C1.1	RA18_YEAST, P10862	BL00518	RAD-18 DNA-binding protein
M01A8.4	BIK1_YEAST, P11709	—	Nuclear fusion protein
C40H1.1	S24577	—	Ovarian protein (<i>D. melanogaster</i>)
C40H1.4	YCS4_YEAST, P25358	BL00030	Yeast hypothetical protein
K04H4.1	CA14_CAEEL, P17139	—	Collagen
C38C10.1	NK3R_RAT, P16177	BL00237	G-protein-coupled receptor
C38C10.5	RGR1_YEAST, P19263	—	Glucose repression regulatory protein RGR1
T26G10.1	DEAD_ECOLI, P23304	BL00039	RNA helicase
T26G10.3	RS24_HUMAN, P16632	BL00529	Ribosomal protein S24
F54C8.1	HCDH_PIG, P00348	BL00067	3-hydroxyacyl-CoA dehydrogenase
F54C8.2	H31_SCHPO, P09988	BL00322	Histone H3
F54C8.4	Y19K_NPVAC, P24656	—	ACMNPV hypothetical protein
F54C8.5	RAS_LENED, P28775	—	Ras family
B0464.1	SYD2_HUMAN, P14868	BL00179	Aspartyl-tRNA synthetase
B0464.5	KCLK_MOUSE, P22518	BL00107	Serine/threonine kinase
F55H2.1	SODC_BOVIN, P00442	BL00087	Superoxide dismutase
F55H2.2	MTPG_SULAC, P22721	—	Membrane-associated ATPase γ -chain
F55H2.5	C561_BOVIN, P10897	—	Cytochrome <i>b₅₆₁</i>
ZK1098.10	TPMX_RAT, P18342	—	Coiled-coil protein
ZK1098.4	GCN3_YEAST, P14741	—	GCN3 (yeast transcription activator)
C48B4.1	CA01_RAT, P07872	BL00072	Acyl-CoA oxidase I
C48B4.2	RHOM_DROME, P20350	—	Rhomboid (<i>D. melanogaster</i>)
C48B4.4	NOD1_RHILO, P23703	BL00211	ATP-binding transport protein
C48B4.5	LIVG_SALTY, P30293	BL00211	ATP-binding transport protein
F58A4.10	UBC7_WHEAT, P25868	BL00183	Ubiquitin-conjugating enzyme
F58A4.3	H3_VOLCA, P08437	BL00322	Histone H3
F58A4.4	PRI1_MOUSE, P20664	—	DNA primase 49K subunit
F58A4.5	RTJK_DROME, P21328	—	Mobile element Jockey-rev. transcriptase
F58A4.7	A36394	BL00038	Transcription factor AP-4
F58A4.8	TBG_XENLA, P23330	BL00227	γ -Tubulin
F58A4.9	RPC9_YEAST, P28000	—	RNA Pol I/III 16K polypeptide
C15H7.2	KFPS_DROME, P18106	BL00790	Tyrosine kinase
C15H7.3	TCPT_HUMAN, P17706	—	Protein tyrosine phosphatase
C07A9.2	G10_XENLA, P12805	—	G10 protein
C07A9.3	KRAC_HUMAN, P31749	BL00107	Ser/Thr kinase
C07A9.4	S20969	BL00470	Na/Ca, K antiporter
C07A9.5	SPCA_DROME, P13395	BL00018	Spectrin α -chain
C07A9.6	UDP2_RAT, P09875	BL00375	UDP-glucuronosyltransferase
T05G5.3	CC2_HUMAN, P06493	BL00107	CDC2 kinase
T05G5.5	S27735	—	Hypothetical protein A (<i>Thermus aquaticus</i>)
T05G5.6	ECHM_RAT, P14604	BL00166	Enoyl-CoA hydratase
T05G5.10	IF5A_HUMAN, P10159	—	Initiation factor 5A
R10E11.1	FSH_DROME, P13709	BL00633	Bromodomain
R10E11.2	VATL_DROME, P23380	BL00605	Vacuolar ATP synthase subunit
R10E11.3	UBP2_YEAST, Q01476	—	Ubiquitin-specific processing protease
R10E11.4	NALS_MOUSE, P15535	—	Galactosyltransferase
ZK632.1	MCM3_YEAST, P24279	—	Mcm 2/3
ZK632.3	S26727	—	Hypothetical protein 186 (<i>Thermoplasma acidophilum</i>)
ZK632.4	MANA_ECOLI, P00946	—	Mannose 6-phosphate isomerase
ZK632.6	CALX_HUMAN, P27824	BL00803	Calnexin
ZK632.8	ARF2_YEAST, P19146	—	ADP-ribosylation factor
K11H3.1	GPDA_DROVI, P07735	—	Glycerol-3-phosphate dehydrogenase
K11H3.3	UCP_HUMAN, P25874	BL00215	Mitochondrial carrier family
ZK757.2	TPCL_HUMAN, P28562	—	Protein tyrosine phosphatase

for pattern formation in a posterior body region³¹. GENE-FINDER predicted a gene candidate with sequence identity to the *mab-5* cDNA sequence (GenBank M22751) on the complementary strand of the cosmid clone C08C3 (annotated as C08C3.3). Approximately 30 kb to the right of *mab-5*, the *abdominal-B*-like gene *egl-5(ceh-11)*^{31,32}, which is required for normal development of several cell types in the tail region, was located (C08C3.1). Interestingly, the *mab-5* and *egl-5* genes are encoded in opposite orientation. A third homeobox gene, *ceh-23*, lies 23 kb to the right of *egl-5* (ZK652.5). The *ceh-23* gene, which is similar to the *Drosophila* genes *Distal-less* and *empty spiracles*, was located by identity to a cDNA clone³². Two additional homeobox genes, *lin-39(ceh-15)* and *ceh-13*, lie approximately 200 kb to the left of *mab-5*. Preliminary sequence data indicate that the order of these two genes relative to *mab-5* is *lin-39*, *ceh-13*, and not *ceh-13*, *lin-39* as originally reported^{32,33}. An unrelated gene, *egl-45*, with no similarity to any known *Drosophila* genes, maps between the *egl-5* and *ceh-23* genes (tentatively correlated to gene candidate C27D11.1), and several other putative genes are contained within the HOM region.

Other genes previously mapped to the region were correlated to distinct loci (shown in parentheses) by genomic DNA sequencing. These include *egl-45* (tentatively C27D11.1), *lin-36* (F44B9.6), *unc-36* (C50C3.11), *unc-86* (C30A5.7), *mig-10* (tentatively F10E9.6), *unc-116* (R05D3.7), *ceh-16* (C13G5.1), *dpy-19* (F22B7.10), *sup-5* (B0523.5), *unc-32* (ZK637.10), *lin-9* (ZK637.7), *gst-1* (R107.7), *lin-12* (LIN12A in cosmid R107), *glp-1* (GLP1A in cosmid F02A9), *emb-9* (K04H4.1), *tbg-1* (F58A4.8) and *ncc-1* (T05G5.3). The *unc-86* and *emb-9* genes and part of the *ceh-16* gene has been sequenced previously^{34,36}. In the cases of *egl-45* (M. Basson and H. R. Horvitz, personal communication), *lin-36* (J. Thomas and H. R. Horvitz, personal communication), *unc-36* (L. Loebel and H. R. Horvitz, personal communication), *unc-116* (ref. 37), *lin-9* (G. Beitel and H. R. Horvitz, personal communication), *gst-1* (ref. 38), *lin-12* (ref. 39) and *glp-1* (ref. 40), cDNA sequences provided by researchers within the *C. elegans* community enabled gene assignments to be made. For *tbg-1* and *ncc-1*, cDNAs from the consortium tag-sequencing project²⁷ allowed assignment to genomic loci. For *mig-10*, *dpy-19* and *unc-32*, transgenic rescue experiments with mutant phenotypes localized the genes to a particular restriction fragment (J. Manser; S. Colloms; D. Thierry-Mieg, personal communications). In some cases, availability of the genomic sequence facilitated this type of analysis.

A few kilobases of genomic DNA sequence which included *sup-5* had been reported previously⁴¹. However, additional genomic sequencing revealed that the *sup-5* locus, a gene for transfer RNA^{Trp}, lies within an intron in the same transcriptional orientation as a homologue of *Drosophila melanogaster flightless I* (ref. 42) (B0303.1). As the *sup-5* mutation has been shown to suppress specific alleles of many unrelated genes⁴³, it is known to be a functional tRNA gene. Further, the *C. elegans fl I* homologue is expressed and spliced as predicted (ref. 42, and R. Wilson, unpublished data).

tRNA genes

The haploid genome of *C. elegans* has been estimated to contain about 300 tRNA genes⁴⁴. Thus, we would expect to find an average of three tRNA genes per megabase of genomic sequence. However, using tRNA^{Scan}⁴⁵, in the 2.181 Mb of the sequence reported here, at least 14 tRNA genes were identified. These are indicated in Fig. 1. Strikingly, two cosmid clones, C14B9 and F22B7, contain seven of these tRNA genes. Like the *sup-5* tRNA gene, a tRNA^{Ser} and two tRNA^{Phe} genes lie within introns of likely genes.

Repeats

Several types of repeated sequence are present in the 2.181-Mb region. Figure 1 indicates the locations of the larger and more complex repeats. Detailed analysis of three major types of

FIG. 1 The region of chromosome III described here. Each line represents 300 kb, with cosmid clones indicated by blue bars; a magenta bar indicates the YAC clone Y53B1. Red and blue arrowheads indicate the approximate starting points of gene candidates for both strands. Circled arrowheads indicate cDNA hits. Red or blue circles indicate the positions of tRNA genes. Green arrowheads indicate the position and type of major repeats. Genetic markers previously mapped to the region and assigned to genomic loci after DNA sequencing are indicated by vertical yellow bars.

METHODS. Subclone libraries were prepared and sequencing reactions performed as previously described^{6,12,14}. For data collection, automated fluorescent DNA sequencers were used. Both the Applied Biosystems 373A and Pharmacia ALF instruments were initially tested, although the 373A was preferred for the shotgun phase because of its greater sample capacity. Both laboratories currently complete two daily runs of each sequencer with 36 lane gels. Thus, with only four of these sequencing instruments, typically more than 1,700 samples are processed per week. After subtracting for failures and contaminating vector sequences, this is sufficient for yearly production of more than 20 Mb of raw sequence data. Two additional sequencing instruments are available for directed sequencing to close gaps and complete the complementary strand. At the conclusion of each sequencer run, the ABI trace files were transferred from the host Macintosh to UNIX workstations for all further work. During the shotgun phase all processing on the UNIX systems is fully automated using a single script. This includes reformatting the trace files using MAKESCF (S.D., unpublished), selection of the quality data from each reading using AUTOTED (L.H., unpublished), clipping off cosmid and sequencing vector sequences using VEP (R.S., unpublished), and assembly using BAP¹³. After the shotgun phase, XBAP¹³, which includes the oligo selection engine of OSP²², is used to edit and finish each project. GENEFINDER and COP (S.D., unpublished) were used to check the finished sequence for errors. GENEFINDER is useful for identifying insertions and deletions in regions containing predicted genes, and COP, which compares the final sequence back to the raw data from which it was produced, is useful for identifying editing errors. We and others have previously described an evaluation of the accuracy of raw data from automated fluorescent DNA sequencers^{6,25}.

repeated sequences (inverted, tandem and interspersed) reveals several interesting features.

Inverted repeats, in which a segment of genomic sequence lies within a few to several hundred bases of an inverted copy of itself, are the most common type of repeat that we have found. Considering only those inverted repeats of up to 1 kb end to end, with at least 70% identity, on average an inverted repeat is found every 5.5 kb. Most of these are quite small, with an average segment length of 70 base pairs (bp) and an average loop size of 164 bp. A relatively high proportion of these repeats (43%) occur in introns, which represent only 20% of the genome. Most inverted repeats fall into families and may be remnants of mobile elements. In particular, there were examples of inverted repeat elements from known transposons Tc3 (ref. 46) and Tc4 (ref. 47).

Tandem repeats, in which a segment of genomic sequence lies adjacent to one or more copies of itself, occur on average every 10 kb. As with inverted repeats, most of these are small, with an average segment length of 17 bp and an average copy number of 14. Interestingly, only 17% of tandem repeats were found in introns, whereas 63% occurred between genes. The most common category of tandem repeats were triplets, some of which were found in predicted exons. One of the most complex tandem repeats found was a 95-bp sequence which was repeated more than 30 times in the clone pAR3 (Fig. 1; 1,144–1,150 kb). This region, which was missing from the cosmid map, had to be recovered from the YAC clone Y53B1 by targeted gap rescue in yeast. Also a large (7.9 kb) tandem repeat, flanked by more complex short repeats, was found in cosmid ZC262 (Fig. 1; 595–633 kb). Sequence assembly for these repeat regions was

accomplished with very stringent parameters and, in some cases, aided by map information.

There are several examples of short repetitive sequences that are scattered throughout the genome, including the 94-bp consensus of the repeat element from most common inverted repeat families, previously observed in *lin-12* and *glp-1* introns^{39,40}. These elements are widely dispersed, with an average of 14 copies every 100 kb at varying levels of conservation. Often, the repeat elements of inverted repeat families are also found in singleton or tandem arrangements. For example, a tandem pair of degenerate elements (69% identical over 77 bp) flanks part of the predicted gene *F58A4.2*. As discussed below, this seems to have been duplicated from a region 200 kb away.

Gene duplications

In addition to the short interspersed repeats discussed above, there are sequences that are repeated tens or hundreds of kilobases apart, with up to 98% similarity. In some cases, these apparent duplications have a complex structure wherein several segments from one region are repeated in a second location, but with different spacings and orientations. Many of these long-range repeats involve coding regions. In particular, there seems to be a recent gene duplication involving *F22B7.5* and *C38C10.4*, which are approximately 750 kb apart but more than 95% similar. The predicted genes *C38C10.3* and *F58A4.2*, mentioned above, are more than 200 kb apart but share a 1.4-kb region that is 98% similar. Two predicted exons are contained within the repeat. If the GENEFINDER predictions are correct, this would be an example of exon shuffling. Perhaps more likely, the *F58A4.2* version is a non-transcribed copy of part of a functional gene in *C38C10.3*. The 7.9-kb repeats in cosmid ZC262 described above contain segments of three different gene candidates, including a kinesin heavy-chain locus which was identified as *unc-116* (ref. 37). The *unc-116* gene begins outside the second copy of the repeat, with the last two exons present in the 7.9-kb. The same two exons in the first copy of the repeat are predicted to splice to a different exon in the second copy of the repeat. In addition to these recent gene duplications, we have identified similarities between other genes in the region. How-

ever, the degree of similarity suggests that these are more ancient in origin and may be examples of new gene families in *C. elegans*.

Prospects

The sequence reported here is already proving useful. In the very narrow sense, several genes previously under study have been sequenced, speeding the analysis and further study of these genes. The full sequence of the homeobox region will clarify its relationship to the *Drosophila Antennapedia* complex. More importantly, the 483 genes identified through genomic sequencing, together with the 1,194 genes discovered in the cDNA tagging project²⁷, are providing new and fruitful avenues for *C. elegans* research.

Furthermore, our experience in the pilot phase indicates that megabase-scale DNA sequencing at a reasonable cost is feasible with current methods and technology^{6,48}. At the same time we feel that significant improvements are possible at almost every step. For example, we have developed an automated DNA template preparation capable of producing 400 M13 templates daily¹⁸. Initial plaque picking can also be done robotically⁴⁹, and instruments are under development that can perform large numbers of small-volume sequencing reactions automatically. Longer read lengths and greater sample capacity for the present generation of fluorescent gel readers are being developed, and more powerful instruments are being designed. Further improvements in the software will soon eliminate much of the sequence editing, which is currently a tedious task. Software tools are already available which simplify the selection of templates and oligonucleotides for directed sequencing (R.S., L.H. and S.D., unpublished). With these improvements and some increase in the scale of effort, production of more than 10 megabases of finished sequence per site per annum seems feasible. With this capacity in both halves of the consortium, the *C. elegans* genome sequence should be essentially completed before the end of 1998. In addition, both laboratories are contributing resources to speed the completion of the *S. cerevisiae* genome sequence. The complete genome sequences of these two organisms will provide insight into the genes that are likely to be common to all eukaryotes, and those specific to metazoans. □

Received 15 November 1993; accepted 5 January 1994.

- Brenner, S. *Genetics* **77**, 71–94 (1974).
- Wood, W. B. et al. *The Nematode Caenorhabditis elegans* (Cold Spring Harbor Laboratory Press, New York, 1988).
- Coulson, A. R., Sulston, J. E., Brenner, S. & Karn, J. *Proc. natn. Acad. Sci. U.S.A.* **83**, 7821–7825 (1986).
- Coulson, A., Waterston, R., Kliff, J., Sulston, J. & Kohara, Y. *Nature* **335**, 184–186 (1988).
- Coulson, A. et al. *Bioessays* **13**, 413–417 (1991).
- Sulston, J. et al. *Nature* **356**, 37–41 (1992).
- Strauss, E. C., Kabori, J. A., Siu, G. & Hood, L. E. *Analyst. Biochem.* **154**, 353–360 (1986).
- Deininger, P. *Analyst. Biochem.* **129**, 216–223 (1983).
- Bankier, A. T. & Barrell, B. G. *Tech. Nucleic Acid Biochem. B* **5**, 1–34 (1983).
- Smith, L. M. et al. *Nature* **321**, 674–679 (1986).
- Connell, C. R. et al. *BioTechniques* **5**, 342–348 (1987).
- Craxton, M. *Methods: A Comparison to Methods in Enzymology* Vol. 3 (ed. Roe, B.) 20–26 (Academic, San Diego, 1991).
- Dear, S. & Staden, R. *Nucleic Acids Res.* **19**, 3907–3911 (1991).
- Halloran, N., Du, Z. & Wilson, R. K. in *Methods in Molecular Biology* Vol. 10: *DNA Sequencing: Laboratory Protocols* (eds Griffin, H. G. & Griffin, A. M.) 297–316 (Humana, Clifton, New Jersey, 1992).
- Schrieffer, L., Gebauer, B. K., Qiu, L. Q., Waterson, R. H. & Wilson, R. K. *Nucleic Acids Res.* **18**, 7455–7456 (1990).
- Lee, L. et al. *Nucleic Acids Res.* **20**, 2471–2483 (1992).
- Hawkins, T. L., Du, Z., Halloran, N. D. & Wilson, R. K. *Electrophoresis* **13**, 552–559 (1992).
- Watson, A., Smaldon, N., Lucke, R. & Hawkins, T. *Nature* **362**, 569–570 (1993).
- Du, Z., Hood, L. & Wilson, R. K. *Meth. Enzym.* **218**, 104–121 (1993).
- Gleeson, T. & Hillier, L. *Nucleic Acids Res.* **19**, 6481–6483 (1991).
- Gleeson, T. J. & Staden, R. *Comput. appl. Biosci.* **7**, 398 (1991).
- Hillier, L. & Green, P. *PCR Meth. Appl.* **1**, 124–128 (1991).
- Dear, S. & Staden, R. *DNA Sequence* **3**, 107–110 (1992).
- Alschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. *J. molec. Biol.* **215**, 403–410 (1990).
- Koop, B. F., Rowan, L., Chen, W.-Q., Deshpande, P., Lee, H. & Hood, L. *BioTechniques* **14**, 442–447 (1993).
- Krause, M. & Hirsh, D. *Cell* **49**, 753–761 (1987).
- Waterston, R. et al. *Nature Genet.* **1**, 114–123 (1992).
- McCombie, W. R. et al. *Nature Genet.* **1**, 124–131 (1992).
- Green, P. et al. *Science* **259**, 1711–1716 (1993).
- Burglin, T. R. et al. *Nature* **351**, 703 (1991).
- Chisholm, A. *Development* **111**, 921–932 (1991).
- Wang, B. B. et al. *Cell* **74**, 29–42 (1993).
- Clark, S., Chisholm, A. & Horvitz, H. R. *Cell* **74**, 43–55 (1993).
- Finney, M., Ruvkun, G. & Horvitz, H. R. *Cell* **55**, 757–769 (1988).
- Guo, X., Johnson, J. J. & Kramer, J. M. *Nature* **349**, 707–709 (1991).
- Naito, M., Kohara, Y. & Kurosawa, Y. *Nucleic Acids Res.* **20**, 2967–2969 (1992).
- Patel, N., Thierry-Mieg, D. & Mancillas, J. R. *Proc. natn. Acad. Sci. U.S.A.* **90**, 9181–9185 (1993).
- Weston, K., Yochem, J. & Greenwald, I. *Nucleic Acids Res.* **17**, 2138 (1989).
- Yochem, J., Weston, K. & Greenwald, I. *Nature* **335**, 547–550 (1988).
- Yochem, J. & Greenwald, I. *Cell* **58**, 553–563 (1989).
- Waterston, R. H. GenBank locus CESUP5 (Acc. no. X54122) (1990).
- Campbell, H. D. et al. *Proc. natn. Acad. Sci. U.S.A.* **90**, 11386–11390 (1993).
- Waterston, R. H. & Brenner, S. *Nature* **275**, 715–719 (1978).
- Sulston, J. E. & Brenner, S. *Genetics* **77**, 95–104 (1974).
- Fichant, G. & Burks, C. J. *molec. Biol.* **220**, 659–671 (1991).
- Collins, J., Forbes, E. & Anderson, P. *Genetics* **121**, 47–55 (1989).
- Yuan, J., Finney, M., Tsung, N. & Horvitz, H. R. *Proc. natn. Acad. Sci. U.S.A.* **88**, 3334–3338 (1991).
- Sulston, J. *Nature* **357**, 106 (1992).
- Uber, D. C., Jaklevic, J. M., Theil, E. H., Lishanskaya, A. & McNeely, M. R. *BioTechniques* **11**, 642–647 (1991).

ACKNOWLEDGEMENTS. We thank T. Schedl for critical reading of the manuscript, P. Kassos and J. Rogers for administrative support, and other members of the *C. elegans* Genome Consortium for technical support. Database searches with St Louis sequences were done remotely using the NCBI BLAST server. ACEDB is available by anonymous ftp from cele.mrc-lmb.cam.ac.uk and from ncbi.nlm.nih.gov. This work was supported by the NIH National Center for Human Genome Research and the MRC Human Genome Mapping Project.

Water-based non-stick hydrophobic coatings

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THERE is a considerable demand for materials that have surfaces to which other substances will not easily stick. Coatings with these properties might be used to make surfaces non-adhesive towards soil, ice, biological foulants, graffiti and other unwanted contaminants. Here we describe a class of water-based non-stick coatings prepared by self-assembly and immobilization of reactive polymeric surfactants^{1,2}. These polymer surfactants contain pendant perfluoroalkyl groups which become oriented so as to yield surfaces with very low energy. Immobilization by crosslinking increases non-stick performance and film toughness. These hard, clear coatings release adhesives and cannot be wetted or attacked by solvents.

Coatings prepared by simply drying solutions of reactive perfluoroalkyl polymeric surfactants (RPPSs) cannot be wetted by liquid hydrocarbons, but are attacked by polar solvents due to the reactive ionic functionality. But when combined and cured (Fig. 1a) with a suitable crosslinking agent, the films cannot be wetted or attacked by any common solvent. This property is easily demonstrated by drawing permanent markers across coated and uncoated areas of polytetrafluoroethylene (Teflon). Ink is easily wiped from coated areas but remains on uncoated areas.

Examples of the reactive ionic functionalities used for crosslinking are carboxylate (COO^-) and phenate ($\text{C}_6\text{H}_5\text{O}^-$); sulphonium or quaternary ammonium groups impart reactive cationic functionality. Crosslinking functionality may either initially possess charge or interact during curing to form intermediate ion-pairs which subsequently react to form covalent bonds. One effective crosslinking approach is to react ammonium-carboxylate functional RPPS with a poly 2-(isopropenyl-2-oxazoline)-based crosslinking agent (poly-IPO). Aqueous formulations containing both materials are stable until applied to a surface. With loss of solvent and ammonia, the carboxylic acid moieties react with the oxazoline ring to form amide-ester crosslinks³ (Fig. 1b). We prepared various RPPSs by copolymerizing acrylate or methacrylate esters of fluoroalkyl alcohols with carboxylic acid functional vinyl monomers. One example^{1,2} is the copolymer of [2-propenoic acid, 2-methyl-2-(heptafluoroethylester)] $\text{CF}_3(\text{CF}_2)_7\text{CH}_2\text{CH}_2\text{OC}(\text{O})\text{C}(\text{CH}_3)=\text{CH}_2$ (monomer A) and β -carboxyethylacrylate [$\text{CH}_2=\text{CHCO}_2\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$] (monomer B). Crosslinking of the final films was adjusted by mixing various amounts of RPPS and poly-IPO having different 'carboxylate to oxazoline mole ratios' ($M_{\text{carb}}/M_{\text{oxa}}$). For example, a larger $M_{\text{carb}}/M_{\text{oxa}}$ value indicates a lower crosslink density.

To study the effect of molecular immobilization by crosslinking, a representative RPPS system was pre-

pared from a 60:40 (weight ratio) copolymer of monomers A and B and crosslinked with varying amounts of an 80:20 (weight ratio) copolymer of IPO and methyl methacrylate. The formulations, comprising a 10:10:80 (weight ratio) of solids, ethylene glycol and water, were cast and cured on microscope slides. These coatings, with different $M_{\text{carb}}/M_{\text{oxa}}$ ratios, were studied using (1) wettability and (2) surface characterization consisting of X-ray photoelectron spectroscopy (XPS), near edge X-ray absorption fine structure (NEXAFS), and scanning force microscopy (SFM).

Wettability⁴⁻⁸ is commonly used to predict intrinsic adhesion^{9,10}. An empirical method for judging wettability is to obtain an 'apparent' or 'classical' contact angle, θ_c , by simply placing liquid drops on a surface. Forcing the liquid to flow yields an 'advancing angle', θ_a ; withdrawing the liquid yields a 'receding angle', θ_r . The difference ($\theta_a - \theta_r$), called 'contact angle' or 'wetting' hysteresis, depends upon surface roughness⁶, heterogeneity⁶, and liquid-induced molecular reorganization¹¹⁻¹³. The 'equilibrium contact angle', θ_e , theoretically has an intermediate value: $\theta_r < \theta_e < \theta_a$. If the hysteresis is zero, then θ_e is equal to θ_a , and θ_e could be used to calculate a type of surface free energy^{14,15}; generally θ_e should not be used because equilibrium equations cannot be applied to nonequilibrium systems.

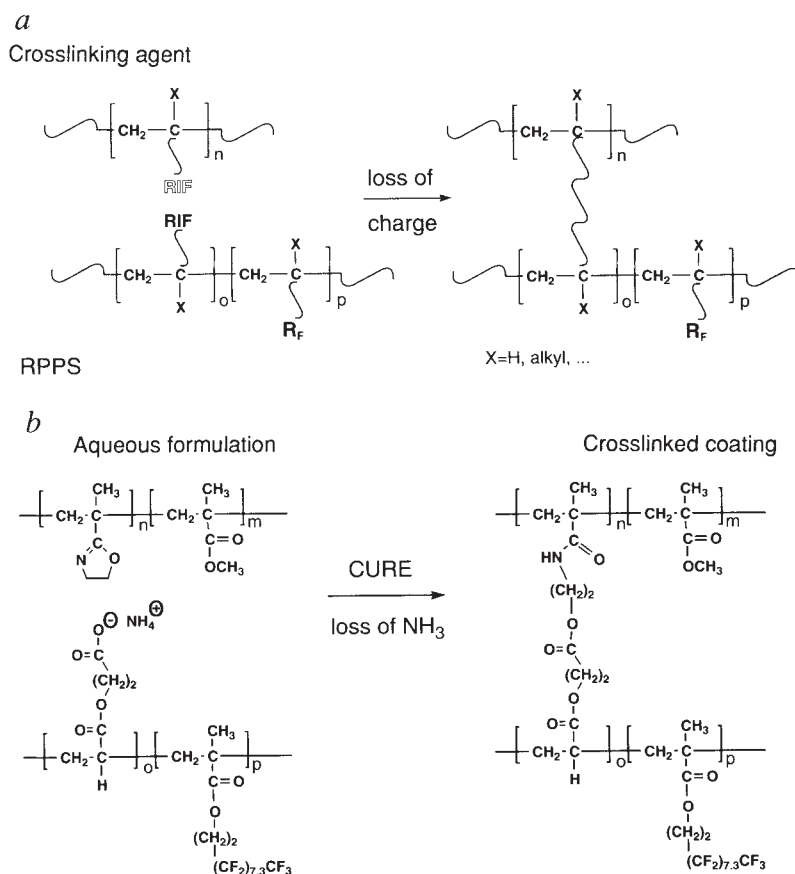
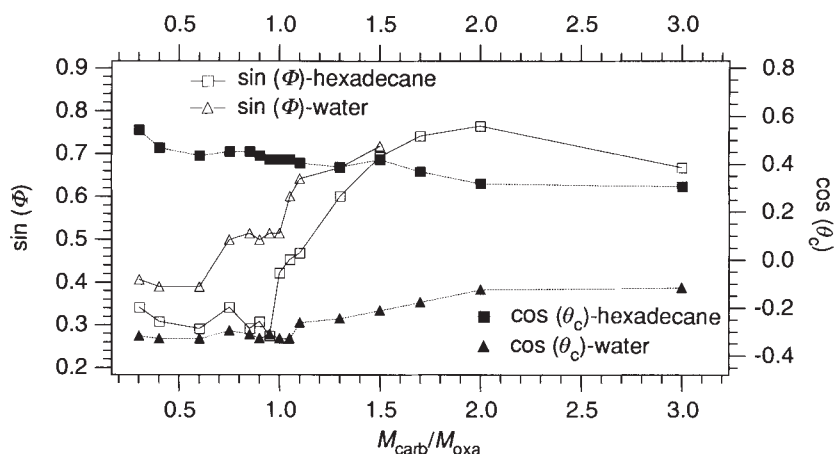


FIG. 1 a, Reactive perfluoroalkyl surfactants (RPPS) react with crosslinking agents, with loss of reactive ionic functionality (RIF), to form crosslinked films (R_F indicates perfluoroalkyl groups). b, The crosslinking reaction of the copolymer of monomers A and B (see text) with poly-IPO.

METHODS. The RPPS copolymer was prepared by peroxide-initiated polymerization^{2,3} followed by neutralization with NH_4OH . Monomer A was obtained from the DuPont Co. (Wilmington, Delaware) and is known as Zonyl TM. The RPPS was purified by dialysis (using Spectra/Por molecular porous membrane tubing); it had a molecular weight cutoff determination of 12,000–14,000 and was titrated using sodium hydroxide to determine the carboxylate activity. The 2-(isopropenyl-2-oxazoline) monomer (monomer B) was obtained from The Dow Chemical Company and the Poly-IPO crosslinker was prepared by peroxide-initiated polymerization followed by purification with dialysis.

FIG. 2 Plot of $\sin(\Phi)$ and $\cos(\theta_c)$ of coatings with varying molar equivalent ratios against $M_{\text{carb}}/M_{\text{oxa}}$, using drops of hexadecane and water. Crosslink density increases with decreasing $M_{\text{carb}}/M_{\text{oxa}}$. (See text for definitions of Φ , θ_c and $M_{\text{carb}}/M_{\text{oxa}}$). The hexadecane (using 5- μl drops) and water (using 50- μl drops) Φ values before and after water exposure (72-h immersion followed by reheating to 110 °C for 1 h) were within an experimental error of $\pm 2^\circ$ except for $M_{\text{carb}}/M_{\text{oxa}}$ between 3.0 and 1.5. All contact and tilt angles were obtained using a Rame-Hart, Model A-100 goniometer equipped with a tilting base (Mountain Lakes, New Jersey). The liquid drops were applied with a Hamilton Microliter Gastight syringe (Reno, Nevada).



The most sensitive empirical method to compare wettability of these coatings is the tilt angle sessile-drop technique^{16–18}. The tilt angle, Φ , is the angle of inclination at which the trailing edge of a drop of known volume will just begin to move. At Φ , the resultant gravitational force (related to $\sin \Phi$) is sufficient to overcome the adhesion between the liquid drop and substrate. At Φ , we measured θ_a (at the leading edge) and θ_r (at the trailing edge) of the drop. The coating's Φ values are a manifestation of the adhesion and hysteresis present and are more sensitive to wettability than θ_c . In order to monitor both the dispersion and polar ('acid-base')⁸ character of the surfaces, we used both hexadecane and water, respectively.

The hexadecane Φ values before and after water exposure (72 h immersion followed by reheating to 110 °C for 1 h) were within an experimental error of $\pm 2^\circ$ except for $M_{\text{carb}}/M_{\text{oxa}}$ between 3.0 and 1.5, which is apparently due to irreversible molecular rearrangement. Reproducibility was obtained if the drops remained on the surface for at least 5 min. The plot of $\sin \Phi$ against $M_{\text{carb}}/M_{\text{oxa}}$ for hexadecane (Fig. 2) indicates a large decrease in wettability (lower Φ) with increased crosslink density, and a minimum Φ at a $M_{\text{carb}}/M_{\text{oxa}}$ ratio of 1. At lower $M_{\text{carb}}/M_{\text{oxa}}$ values (excess crosslinking agent) there is a slight increase in hexadecane wettability. Hysteresis ($\theta_a - \theta_r$) also decreases to a minimum value of only 1° – 2° at $M_{\text{carb}}/M_{\text{oxa}}$ between 0.5 to 1.2, which suggests that in this case surface roughness is not the cause of hysteresis^{11–13}. The surface free energy, calculated from the Good–Girfalco–Fowkes^{14,15} equation using hexadecane θ_c at an $M_{\text{carb}}/M_{\text{oxa}}$ ratio of 1, is 14 mJ m^{-2} . The plot of $\cos \theta_c$ against $M_{\text{carb}}/M_{\text{oxa}}$ for hexadecane (Fig. 2) shows an increase in wettability with decreasing $M_{\text{carb}}/M_{\text{oxa}}$ ratios rather than the decrease in wettability by tilt angles. This is consistent with both lower surface densities of perfluoroalkyl groups at lower $M_{\text{carb}}/M_{\text{oxa}}$, and the inability of oriented, immobilized perfluoroalkyl groups to reorganize during the short time the nonpolar advancing liquid drop contacts the surface.

The Φ values before and after 72 h water exposures were within experimental error of $\pm 2^\circ$ except for $M_{\text{carb}}/M_{\text{oxa}}$ ratios between 3.0 and 1.7. For these samples the crosslink density is apparently too low to immobilize the surface molecules at the interface. A plot of $\sin \Phi$ against $M_{\text{carb}}/M_{\text{oxa}}$ (Fig. 2) illustrates that water wettability decreases with increased crosslink density; excess crosslinking agent ($M_{\text{carb}}/M_{\text{oxa}} > 1$) further decreases wettability. The plot of $\cos \theta_c$ also shows a decrease in wettability with increased crosslinking agent in contrast to increased wettability using hexadecane. Because water strongly interacts with polar groups, it alters the surface molecular structure more than hexadecane. Thus, crosslinking decreases wettability with

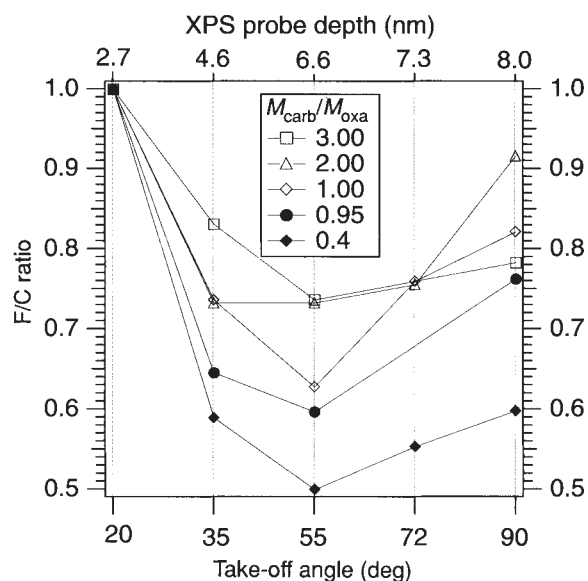


FIG. 3 Normalized fluorine/carbon (F/C) ratios (obtained by setting F/C equal to 1.00 for data at 20° take-off angle) using X-ray photoelectron spectroscopy (XPS) for films with different $M_{\text{carb}}/M_{\text{oxa}}$ ratios, shown as a function of probe depth. (Concentrations of F and C in atom%). For comparing relative F/C gradients, the F/C ratio curves were shifted so the initial (20° take-off angle) values were set to 1. The actual F/C ratios were all within $\pm 20\%$ for varying $M_{\text{carb}}/M_{\text{oxa}}$ due to compositional inhomogeneities in the probed area. The unshifted F/C ratios for $M_{\text{carb}}/M_{\text{oxa}}$ of 3.00, 2.00, 1.00, 0.95 and 0.40 were 0.95, 1.31, 0.91, 1.29 and 1.12, respectively.

METHODS. The analyses were performed on a Fisons Instruments Model S Probe X-ray photoelectron spectrometer using a monochromatic Al K α X-ray source. Spectra were collected using a spot size of $100 \times 800 \mu\text{m}$ and a spectrometer energy resolution of 0.7 eV (Au 4f_{7/2}). Because the samples were insulating, charge compensation using electrons (4 eV energy) was necessary. For each sample analysed, the take-off angle (the angle between the surface plane and detector) was varied between 20° and 90°. Radiation-induced degradation²⁶ (decrease in the F/C ratio) was observed during XPS measurements in the fluorinated polymers. The degradation rate was used to correct each F/C ratio. Most of the degradation was determined to be due to electron bombardment during charge compensation.

water more than with hexadecane. Contact angle hysteresis also decreases with increasing crosslinking, but is larger than obtained from hexadecane.

We used carbon K-edge NEXAFS spectroscopy (using linearly polarized synchrotron radiation at the National Synch-

rotron Light Source (UIA beamline) at Brookhaven National Laboratory) to measure the orientation (in vacuum) of the surface perfluoroalkyl groups in the top 3 nm of the surface^{19,20}. Orientation was determined from the relative angular dependence of the X-ray absorption cross-section by monitoring the partial electron yield¹⁹. More perfluoroalkyl orientation was observed in films having lower crosslink density. We determined from the polarization angular dependence of the C–F resonances (in the CF₂ groups) that the perfluoroalkyl groups are oriented from the surface at an average angle of ~58° and ~48° for $M_{\text{carb}}/M_{\text{oxa}}$ values of 3.0 and 0.95, respectively. Orientation of closely packed perfluoroalkyl groups yields low wettability²¹. But the apparent surface energy can increase, if exposure to solvents (or adhesives) induces surface reorientation. Thus, the hexadecane $\cos \theta_c$ increases, but $\sin \Phi$ decreases, with increasing crosslinking agent (Fig. 2).

Angle-resolved XPS was used to measure the fluorine to carbon (F/C) ratios as a function of probe depth in the first 10 nm of the film surfaces for the series of crosslinked coatings. The F/C gradient was determined by varying the angle between the surface plane and the detector²² (take-off angle). The total depth probed, as a function of take-off angle, was estimated using a photoelectron escape depth of 4.0 nm (± 1.5 nm). We observed systematic changes in the normalized F/C ratio that depend upon $M_{\text{carb}}/M_{\text{oxa}}$ (Fig. 3). These ratios as a function of probe depth with varying $M_{\text{carb}}/M_{\text{oxa}}$ indicate minimum ratios at ~6.6 nm and increasing ratios from 6.6–8.0 nm. This indicates a second layer of perfluoroalkyl groups ~8.0 nm below the surface, and is consistent with a perfluoroalkyl layered structure.

Surface morphology on the nanometre scale was obtained using the SFM²³ (Nanoscope II, Digital Instruments, Inc.) to determine if surface roughness correlated with the $M_{\text{carb}}/M_{\text{oxa}}$ (crosslink density). The root-mean-square (r.m.s.) roughness values for SFM images (1 $\mu\text{m} \times 1 \mu\text{m}$ area) for $M_{\text{carb}}/M_{\text{oxa}}$ ratios of 3.00, 2.00, 1.00, 0.95 and 0.40 are 0.5 nm, 0.76 nm, 0.42 nm, 1.3 nm and 0.36 nm, respectively. We found no correlation between the wettability behaviour and surface morphology or roughness.

Polar solvents (such as water) are needed to effectively orient the polymer surfactants²⁴. Extensive wettability studies indicate that high-boiling co-solvents such as ethylene glycol dramatically improves non-stick performance, apparently by promoting molecular mobility²⁵ and intermolecular packing in the final stages of curing. Curing in high humidity also increases non-stick performance. The relative rate and extent of crosslinking (monitored by infrared spectroscopy) increases with the addition of co-solvents. □

24. Adamson, A. W. *Physical Chemistry of Surfaces* 4th edn 63–64 (Wiley, New York, 1982).
25. Owen, M. J. *Comments inorg. Chem.* **7**, 195–213 (1988).
26. Chaney, R. & Barth, G. Z. *analyt. Chem.* **329**, 143–146 (1987).

ACKNOWLEDGEMENTS. The authors thank J. J. Curphy, G. E. Mitchell, M. J. Radler, E. G. Rightor and J. L. Gland for assistance with NEXAFS, and R. A. Nyquist for assistance with infrared studies of the reaction mechanism. The molecular modelling calculations were carried out by N. G. Rondan. We also thank the following for their contributions; P. McCrackin, R. F. Harris, G. Rose, D. M. Pickelman, M. K. Chaudhury and J. F. Schmidt. Certain commercial names are identified in this paper for the purpose of clarity in the presentation. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology.

Role of the product in the transformation of a catalyst to its active state

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Oxide catalysts, which are used in a broad range of important industrial processes^{1–4}, are generally prepared in the form of a precursor which is converted into the active catalytic form only under well defined reaction conditions. A vanadium phosphorus oxide catalyst is used to effect selective oxidation of *n*-butane to maleic anhydride: (VO)₂P₂O₇ is considered^{5–7} to be the main active component, and is prepared from the precursor VOHPO₄·0.5H₂O. Here we use *in situ* Raman spectroscopy to study the conversion of this precursor to the active form in real time. We find that, during this process, the crystalline structure of VOHPO₄·0.5H₂O becomes totally disordered at the same time as selectivity for maleic anhydride becomes apparent. Furthermore, our results indicate that this product seems to play a role in bringing about this change; thus, it seems that the selective reaction product assists in the creation of the active sites. We suggest that this phenomenon might be quite general in oxide catalysis.

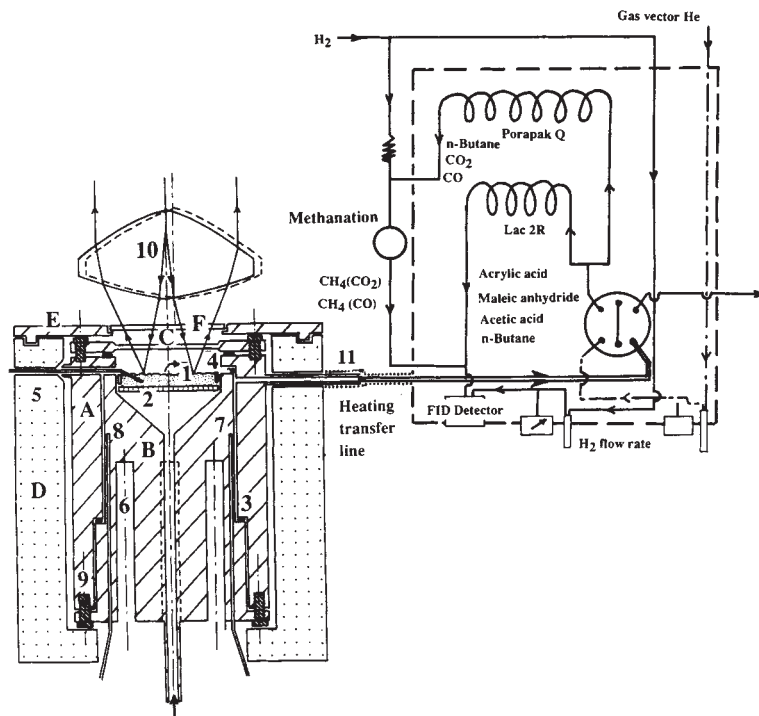
There have been few real-time *in situ* studies of the transformation of the oxide catalyst precursor to the final active form, yet knowledge of this important process would aid our understanding of how and why these catalysts function. One reason is that techniques readily available⁸ to study this process are not well suited to oxides. But we have previously shown⁹ that Raman spectroscopy is well suited to the study of vanadium phosphorus oxides, because vanadium phosphorus oxide compounds exhibit characteristic bands in the Raman spectrum. Furthermore, we have constructed an *in situ* reaction cell⁹ and have found that the Raman spectrum remains sufficiently well defined at elevated temperature to allow characterization of phases in the catalyst. Standard vanadium phosphorus oxide compounds and catalysts are usually prepared using non-aqueous solvents, for example butanol¹⁰, at some stage of the procedure. Solvent molecules are retained in the crystalline structure and their presence causes marked fluorescence in the laser beam, and so these materials are unsuitable for detailed *in situ* Raman spectroscopic study until the decomposition temperature—where this fluorescence vanishes—is reached. We have found, however, that VOHPO₄·0.5H₂O prepared using an aqueous medium does not give rise to this problem. Hence an investigation of vanadium phosphorus oxides using *in situ* Raman spectroscopy should be ideal for a detailed real time study of the conversion of the catalyst precursor to the final catalyst.

Received 1 November 1993; accepted 13 January 1994.

1. Schmidt, D. L., McCrackin, P. J. & Coburn, C. E. US Patent No. 4929666 (1990).
2. Schmidt, D. L., Coburn, C. E. & McCrackin, P. J. US Patent No. 5006624 (1991).
3. Frump, J. A. *Chem. Rev.* **71**, 483–505 (1971).
4. deGennes, P. G. *Rev. mod. Phys.* **57**, 827–863 (1985).
5. Berg, J. C. *Wettability* (Decker, New York, 1993).
6. Johnson, R. E. Jr & Dettre, R. H. in *Surface and Colloid Science* Vol. 2 (ed. Matijević, E.) 85–153 (Wiley-Interscience, New York, 1969).
7. Fowkes, F. M., Kaczinski, M. B. & Dwight, D. W. *Langmuir* **7**, 2464–2470 (1991).
8. Mittal, K. L. & Anderson, H. R. Jr *Acid-Base Interactions: Relevance to Adhesion Science and Technology* (VSP Utrecht, 1991).
9. Kinloch, A. J. *Adhesion and Adhesives Science and Technology* Ch. 3 (Chapman & Hall, New York, 1987).
10. Pocius, A. V. in *Structural Adhesives Chemistry and Technology* Ch. 1 (ed. Hartshorn, S. R.) (Plenum, New York, 1986).
11. Chen, Y. L., Helm, C. A. & Israelachvili, J. N. *J. phys. Chem.* **95**, 10736–10747 (1991).
12. Yoshizawa, H., Chen, Y. L. & Israelachvili, J. N. *J. phys. Chem.* **97**, 4128–4140 (1993).
13. Chaudhury, M. K. & Owen, M. J. *J. phys. Chem.* **97**, 5722–5726 (1993).
14. Fowkes, F. M. *Ind. Engng Chem.* **56**, 40–52 (1964).
15. Good, R. J. & Girfalco, L. A. *J. phys. Chem.* **64**, 561–565 (1960).
16. Bikerman, J. J. *J. Colloid Sci.* **5**, 349–359 (1950).
17. Furmidge, C. G. L. *J. Colloid Sci.* **17**, 309–324 (1962).
18. Vogler, E. A. *Langmuir* **8**, 2013–2020 (1992).
19. Stöhr, J. *NEXAFS Spectroscopy* (Springer, Berlin, 1992).
20. Mitchell, G. E. et al. in *Polymer and Inorganic Interfaces* (eds Opila, R. L., Czanderna, A. W. & Boerio, F. J.) 215–220 (Symp. Proc. No. 304, Mater. Res. Soc., Pittsburgh, 1993).
21. Shafirin, E. G. & Zisman, W. A. *J. phys. Chem.* **64**, 519–524 (1960).
22. Briggs, D. & Seah, M. (eds) *Practical Surface Analysis*, Vol. 1—Auger and X-Ray Photoelectron Spectroscopy (Wiley, New York, 1990).
23. Meyers, G. F., DeKoven, B. M. & Seitz, J. T. *Langmuir* **8**, 2330–2335 (1992).

FIG. 1 Design of *in situ* Raman cell with analysis of products by gas chromatography (GC). Key: (A) chamber upper part; (B) chamber lower part; (C) glass window; (D) isolating shell; (E) thermal screen; (F) anticaloric filter; (1) catalyst; (2) sintered glass; (3 and 4) tightening rings; (5) temperature controller; (6) heater; (7) chamber heating controller; (8) thermal security; (9) mounting bolts; (10) rotating lens; (11) heated transfer line.

METHODS. The catalyst precursor ($\text{VOHPO}_4 \cdot 0.5\text{H}_2\text{O}$) was prepared by the following procedure. V_2O_5 (Analar grade, 7.26 g) was dissolved in aqueous HCl (37%, Analar, 80 ml) and refluxed for 3 h. Aqueous H_3PO_4 (85%, Analar, 10.70 g) was added and the solution was refluxed for a further 3 h, and then the solvent was carefully removed to yield a blue-green solid. This solid was then refluxed with H_2O (10 ml g^{-1}) and filtered hot to remove trace impurities of $\text{VO}(\text{H}_2\text{PO}_4)_2$. The resultant blue solid was dried (120°C , 16 h) and confirmed by X-ray diffraction to be $\text{VOHPO}_4 \cdot 0.5\text{H}_2\text{O}$. This catalyst precursor (1.5 g) was placed in the *in situ* Raman cell and the temperature was controlled by a thermocouple placed directly in the centre of the catalyst powder. Reaction gas (1.5% *n*-butane in air) was flowed through the catalyst powder at a gas hourly space velocity of $2,000 \text{ h}^{-1}$ (that is, 2,000 volumes of gas per unit volume of catalyst per h). The effluent gas from the reactor was analysed using on-line GC with a flame ionization detector, together with a methanator to enable analysis of CO and CO_2 . Laser Raman spectra were recorded using a DILOR OMARS 89 spectrophotometer equipped with an intensified photodiode array detector. The emission line at 514.5 nm from Ar^+ ion laser was used for excitation. The power of the incident beam was 30 mW and the time of acquisition was adjusted according to the intensity of the Raman scattering. To improve signal-to-noise ratio, spectra were accumulated. To reduce both thermal and photodegradation of samples, the laser beam was scanned on the sample surface by means of a rotating lens (ref. 12). The scattered light was collected



in the back scattering geometry. The presence of the different V-P-O structures in the catalyst was determined from the Raman spectrum of pure V-P-O reference phases recorded at the same temperature and published elsewhere⁹.

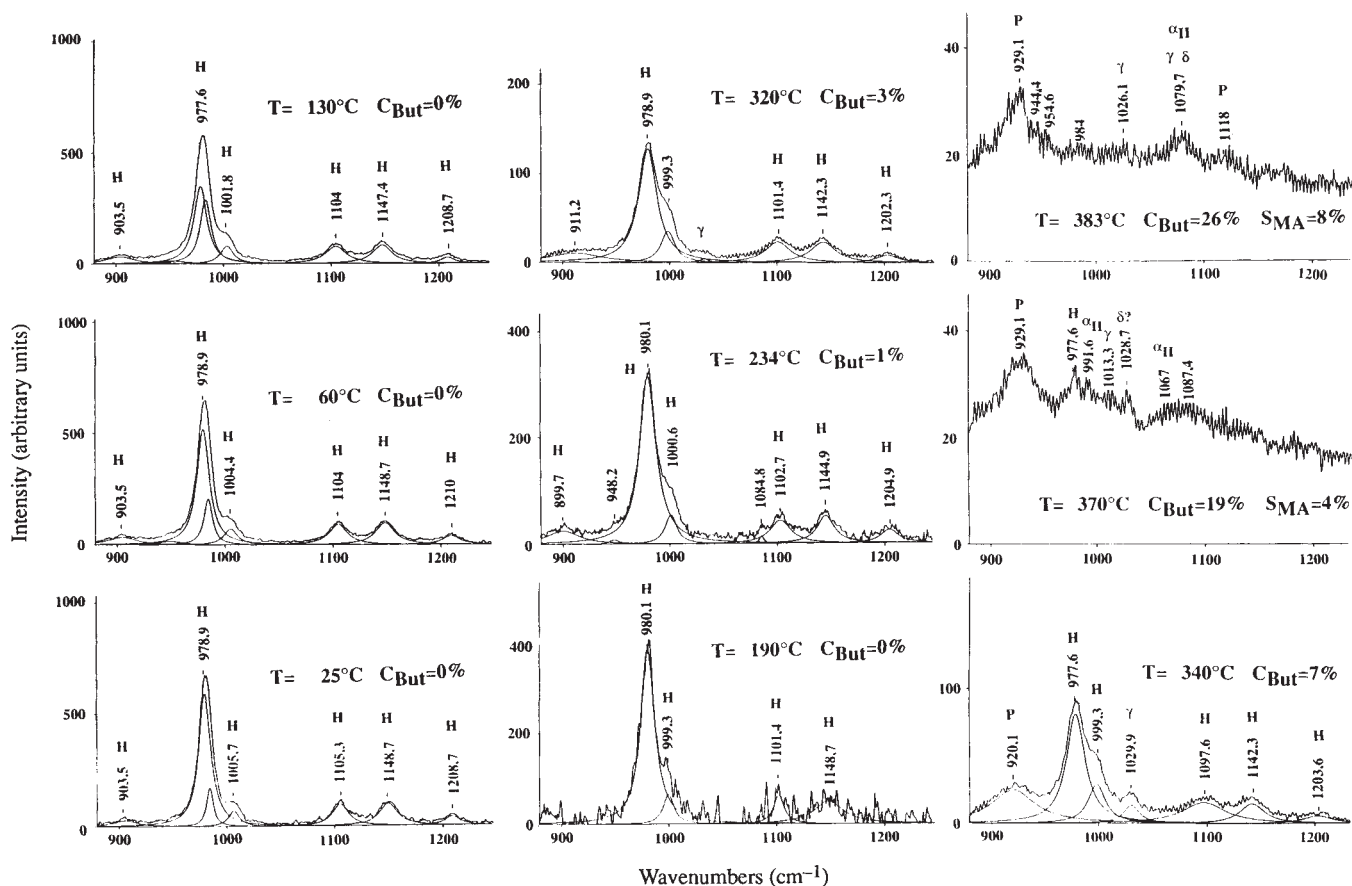
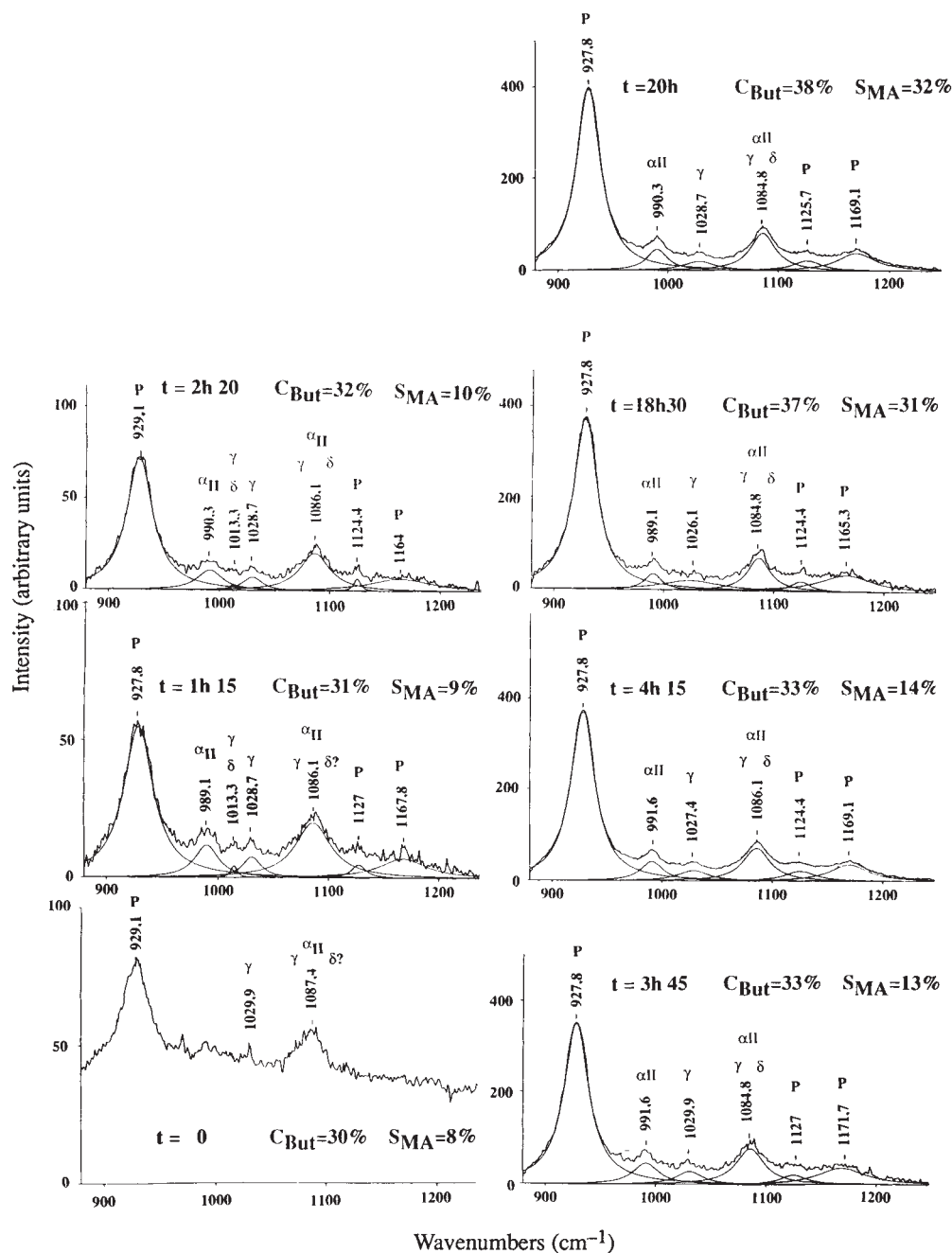


FIG. 3 *In situ* Raman spectra and catalytic performance for *n*-butane oxidation to maleic anhydride (MA) during the transformation of $\text{VOHPO}_4 \cdot 0.5\text{H}_2\text{O}$ under *n*-butane/air (1.5%) at 394 °C as function of time (*t*) (symbols used in peak assignments, as in Fig. 2).



The catalyst precursor $\text{VOHPO}_4 \cdot 0.5\text{H}_2\text{O}$ (1.5 g) was placed in the *in situ* Raman cell, which is shown schematically in Fig. 1. The temperature of the catalyst was slowly raised ($1.25^\circ\text{C min}^{-1}$) and laser Raman spectra were recorded with simultaneous product analysis (Fig. 2). At 25°C , the Raman spectra of the well crystalline $\text{VOHPO}_4 \cdot 0.5\text{H}_2\text{O}$ precursor was well defined. On heating, no significant changes in the spectra were observed up to 340°C , although the intensity of the Raman

scattering decreased. Conversion of *n*-butane was obtained at 234°C , but only non-selective products (CO and CO_2) were observed up to 340°C . At 370°C , the intensity of the Raman scattering significantly decreased, and a large number of V-P-O compounds were observed in this complex structure. However, three structural features were observed: (1) the main band of precursor ($\text{VOHPO}_4 \cdot 0.5\text{H}_2\text{O}$) at 978 cm^{-1} was vanishing (completely absent at 380°C); (2) the characteristic band of the pyrophosphate $(\text{VO})_2\text{P}_2\text{O}_7$ at 929 cm^{-1} was now the main band; (3) the appearance at 370°C of the $1,087\text{ cm}^{-1}$ band by itself revealed the presence of VOPO_4 , since α_{II} , γ - and δ - VOPO_4 exhibit such a feature. Although the presence of δ - VOPO_4 could not be ruled out, the 990 (α_{II}) and $1,013$ and $1,029\text{ cm}^{-1}$ (γ) bands appearing at 394°C clearly showed that α_{II} - and γ - VOPO_4 had been formed⁹.

The appearance at 370°C of vanadium(v) phosphate phases were correlated with the onset of maleic anhydride production and the high degree of disorder is observed only when maleic anhydride is first observed. Furthermore, Raman spectra of the

FIG. 2 *In situ* Raman spectra and catalytic performance for *n*-butane oxidation to maleic anhydride (MA) during the transformation of $\text{VOHPO}_4 \cdot 0.5\text{H}_2\text{O}$ under *n*-butane/air (1.5%). In each panel, *T* indicates the temperature of the sample, C_{But} indicates the conversion of butane and S_{MA} indicates the selectivity to maleic anhydride (fraction of the converted butane molecules transformed into MA) observed at the reaction conditions. The peaks in the Raman spectra are assigned as follows: H, $\text{VOHPO}_4 \cdot 0.5\text{H}_2\text{O}$; P, $(\text{VO})_2\text{P}_2\text{O}_7$; α_{II} , α_{II} - VOPO_4 ; γ , γ - VOPO_4 ; δ , δ - VOPO_4 .

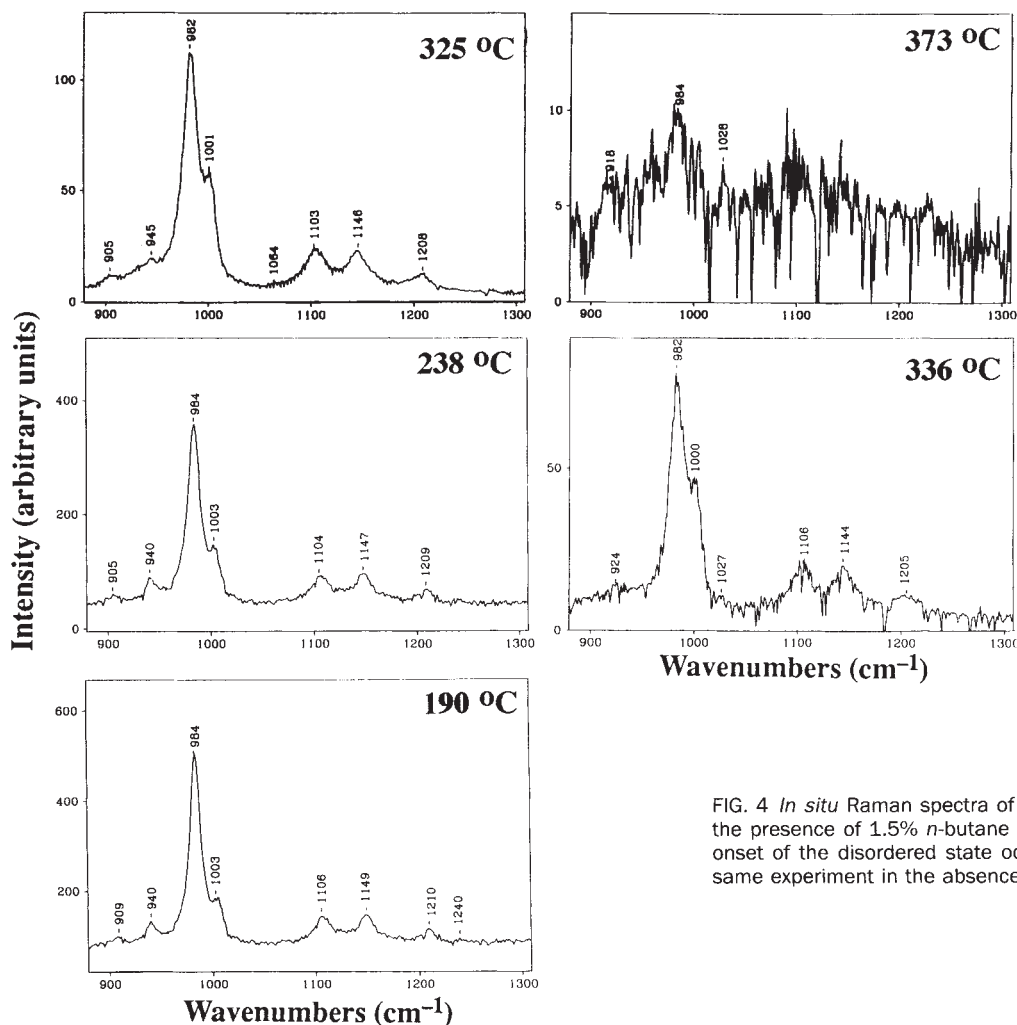


FIG. 4 *In situ* Raman spectra of the transformation of $\text{VOHPO}_4 \cdot \text{H}_2\text{O}$ in the presence of 1.5% *n*-butane in air +23 torr H_2O showing that the onset of the disordered state occurs at a similar temperature to the same experiment in the absence of H_2O (Fig. 2).

V-P-O compounds present in the final catalyst (as shown in a separate study) were found not to exhibit a loss of Raman scattering owing to disorder under identical reaction conditions⁹. It is therefore apparent that the conversion of the oxide precursor to the final catalyst structure is not a simple process in which one structure recrystallizes to give another.

Once the required reaction temperature was attained (394 °C), it was maintained for 20 h and Raman spectra were recorded at suitable intervals (Fig. 3). During this time, the conversion of *n*-butane, and particularly the selectivity of this conversion to maleic anhydride, significantly increased and the intensity of the Raman scattering increased as the active catalyst components gradually crystallized under reaction conditions, giving Raman spectra characteristic of $(\text{VO})_2\text{P}_2\text{O}_7$, α - VOPO_4 , γ - and possibly δ - VOPO_4 . The selectivity and conversion data were confirmed by an identical test using a standard laboratory microreactor, indicating that the reaction data obtained during the activation in the *in situ* cell are representative of this catalyst and are in agreement with published data¹⁰.

The correlation between catalyst disordering at 370 °C and the appearance of maleic anhydride implies that the latter may be responsible for the former. Other interpretations are unlikely for the following reasons. First, the presence of the reactants and products is considered to be crucial to obtain the required phase transformations, because if the precursor is heated in N_2 alone it transforms to $(\text{VO})_2\text{P}_2\text{O}_7$ only, or in air in the absence of *n*-butane it changes to γ - VOPO_4 (ref. 9). Second, it is possible that the presence of water from the non-selective oxidation process could be responsible for the structural changes rather than

the presence of maleic anhydride. We therefore did two further experiments. In the first the catalyst precursor was treated in the Raman cell *in situ* as already described, except that H_2O vapour (23 torr) was added to the 1.5% *n*-butane/air reactant at 120 °C using a saturator at 24.5 °C. On heating, the observed structural changes and onset of the disordered state did not appear to be significantly affected by the presence of additional H_2O (Fig. 4), although the final catalyst contained proportionately more $(\text{VO})_2\text{P}_2\text{O}_7$ than in the absence of added H_2O . In the second experiment, the procedure was the same except that now maleic anhydride vapour (54 torr) was added to the reactants at 185 °C using a saturator at 120 °C. Control experiments showed that there was no maleic anhydride condensation on the cell window. In this case, the presence of added anhydride decreased the temperature at which the disordered state was observed and this transition now started below 300 °C (Fig. 5), and the final catalyst contained proportionately more α - VOPO_4 than in the absence of added anhydride. This result shows that the presence of the products, in particular maleic anhydride, is an important factor in controlling the structural transformations in the V-P-O catalyst system.

Our study demonstrates the efficacy on *in situ* Raman spectroscopy for the real time study of oxide catalyst activation; it also shows that this process is complex. In particular, the loss of catalyst structure, together with the onset of the generation of the desired product, indicates that the creation of the surface active sites is highly dependent on the nature of surface carbonaceous species formed during the reaction. Our results show that the creation of the active surface may not be solely dependent

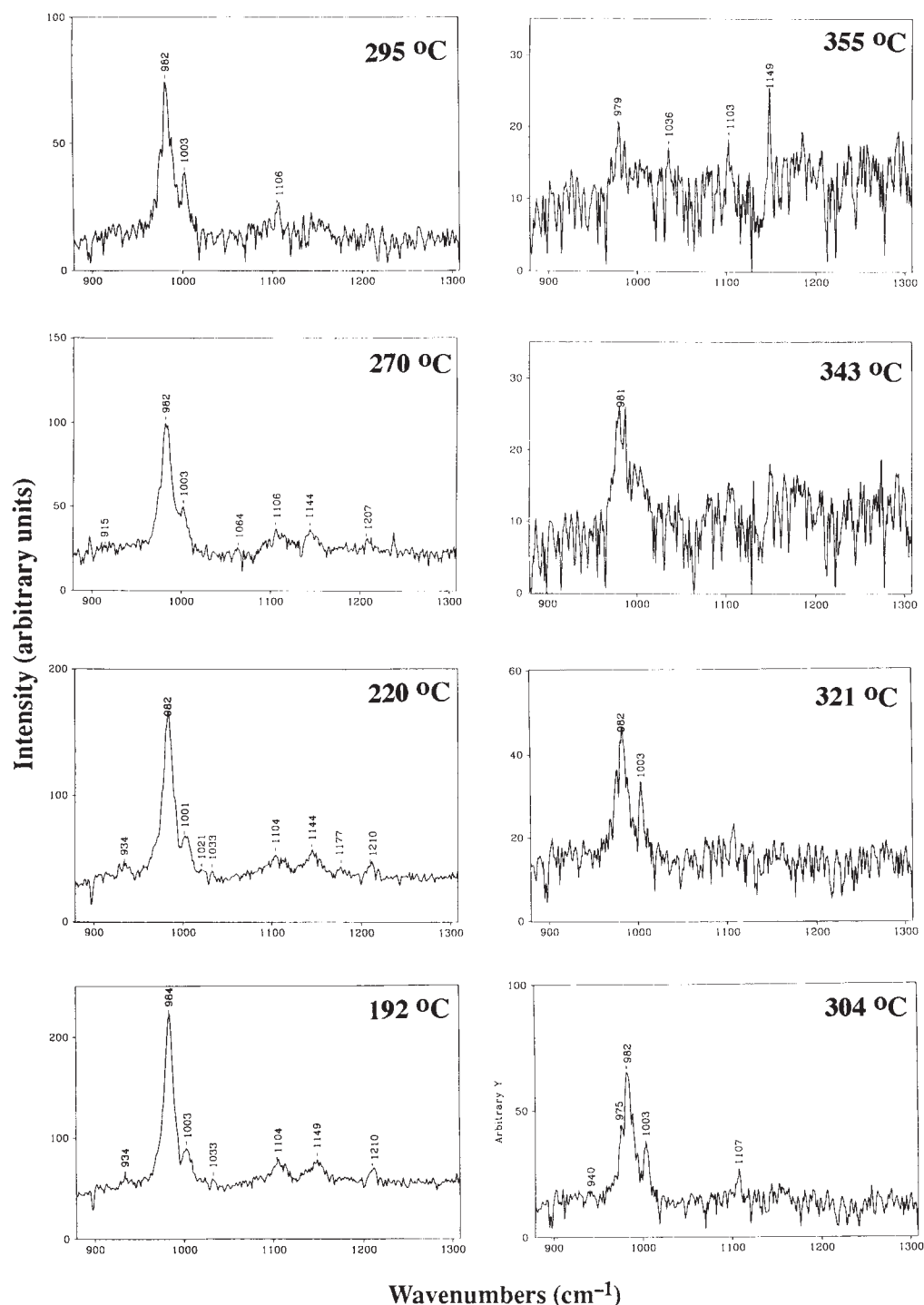


FIG. 5 *In situ* Raman spectra of the transformation of $\text{VOHPO}_4 \cdot 0.5\text{H}_2\text{O}$ in the presence of 1.5% *n*-butane in air +54 torr maleic anhydride (MA), showing that the onset of the disordered state starts at $<300^\circ\text{C}$.

on the nature of the bulk catalyst phases. In this way, the active site is specifically formed under the reaction conditions and requires the presence of the reacting molecules and products that may provide a template to enable surface structures of the catalyst to crystallize. We have observed similar results for the trans-

formation of the $\text{VO}(\text{H}_2\text{PO}_4)_2$ to $\text{VO}(\text{PO}_3)_2$ in the presence of *n*-butane/air. We propose that these results are important to oxide catalysts for oxidation reactions in general, and suggest that the catalyst precursor to final catalyst transformation warrants further attention. □

Received 5 November; accepted 21 December 1993.

1. Bond, G. C. *Heterogeneous Catalysis: Principles and Applications* (Clarendon, Oxford, 1987).
2. Higgins, R. & Hayden, P. *Catalysis* **166** (Specialist Period. Rep., Royal Soc. of Chem., London, 1977).
3. Parkyn, N. D., Warburton, C. I. & Wilson, J. D. *Catal. Today* **18**, 385–442 (1993).
4. Bond, G. C., Flamerz, S. & Shukri, R. *Faraday Discuss. chem. Soc.* **87**, 65–77 (1989).
5. Centi, G. *Catal. Today* **16**, 5–26 (1993).
6. Hodnett, B. K. (ed.) *Catal. Today* **1** (5) (1987).
7. Centi, G., Trifiro, F., Ebner, J. R. & Franchetti, V. M. *Chem. Rev.* **68**, 55–80 (1988).
8. Burch, R. *Catal. Today* **9**, 1–235 (1991).

9. Ben Abdelouahab, F., Olier, R., Guilhaume, N., Lefebvre, F. & Volta, J. C. *J. Catal.* **134**, 151–167 (1992).
10. Johnston, J. W., Johnston, D. C., Jacobson, A. J. & Brody, J. F. *J. Am. Chem. Soc.* **106**, 8123–8128 (1984).
11. Hutchings, G. J. *Appl. Catal.* **72**, 1–32 (1991).
12. Zimmerer, N. & Kiefer, W. *Appl. Spectrosc.* **28**, 279–281 (1974).

ACKNOWLEDGEMENT. We thank M. Rouillet for the GC calibration. G.J.H. thanks the French CIES for financial support.

Generic modelling of cooperative growth patterns in bacterial colonies

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BACTERIAL colonies must often cope with unfavourable environmental conditions^{1,2}. To do so, they have developed sophisticated modes of cooperative behaviour³⁻¹⁰. It has been found that such behaviour can cause bacterial colonies to exhibit complex growth patterns similar to those observed during non-equilibrium growth processes in non-living systems¹¹; some of the qualitative features of the latter may be invoked to account for the complex patterns of bacterial growth¹²⁻¹⁸. Here we show that a simple model of bacterial growth can reproduce the salient features of the observed growth patterns. The model incorporates random walkers, representing aggregates of bacteria, which move in response to gradients in nutrient concentration and communicate with each other by means of chemotactic 'feedback'. These simple features allow the colony to respond efficiently to adverse growth conditions, and generate self-organization over a wide range of length scales.

We have grown bacterial colonies under different growth conditions^{12,13} ranging from a very low level of nutrient (0.1 g peptone per litre) to a very rich mixture (10 g peptone per litre), and from a soft substrate (~1% agar concentration) to a hard substrate (4% agar concentration). Growth was started with a droplet (5 µl containing ~10⁵ bacteria) inoculation at the centre of Petri dishes incubated at 37 °C and 30% humidity. The growth pattern we describe are of bacteria derived from *Bacillus subtilis* strain 168 (refs 12, 13). The colonies adopt various shapes as growth conditions are varied (Fig. 1): patterns are compact at high peptone concentrations and become more ramified at low peptone concentrations (0.5 g l⁻¹), in agreement with results reported in refs 12-18. Surprisingly, at <~0.25 g peptone per litre, colonies adopt a more organized (well defined circular envelope), dense structure (Fig. 1d).

Optical microscopy reveals that the bacteria perform a random walk-like movement within a well defined envelope. The latter (Fig. 2) is formed presumably by chemicals that are excreted by the bacteria and/or by fluid drawn by the bacteria from the agar. The envelope propagates slowly as if by the action of effective internal pressure produced by the collective movement of the bacteria. At very low peptone concentrations the density of bacteria is low—the distance between bacteria is up to several times the size of an individual bacterium. In this range, the bacteria are longer (~5 µm) (Fig. 2e) and the movement seems to be more organized. At high agar concentration there is a boundary layer of high bacterial density at the leading tips of the growing branches (Fig. 2f). In this range, colonies display a pronounced structure in the perpendicular direction as well (Fig. 2c).

The growth of bacterial colonies presents an inherent additional level of complexity compared to non-living systems, as the building blocks themselves are living systems^{15,19,20}, each having its own autonomous (at times 'selfish') self-interest and internal degrees of freedom. To model the growth, we included the following generic features: (1) diffusion of nutrients; (2) movement of the bacteria; (3) reproduction and sporulation; (4) local communication. Diffusion of nutrients is handled by solving the

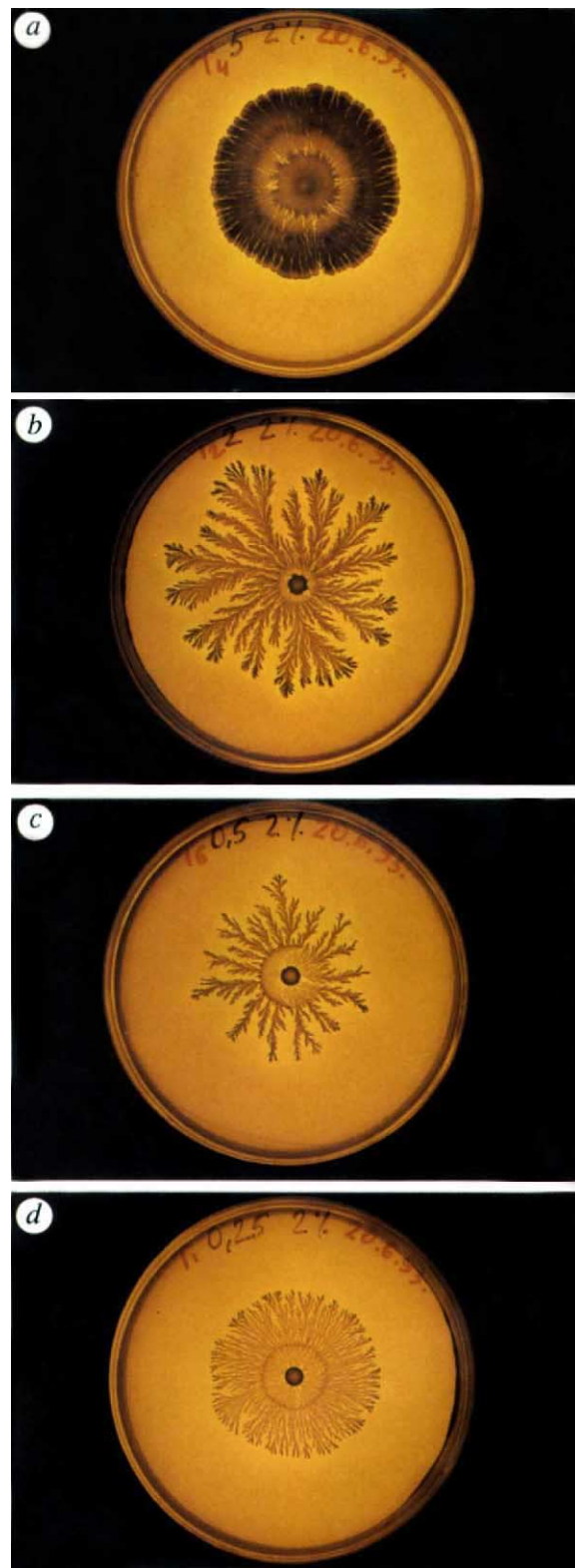


FIG. 1 Observed patterns of colonies grown on a substrate with 2% agar concentration. The peptone level is 5, 2, 0.5 and 0.25 g l⁻¹ for a, b, c and d respectively. At high peptone levels the branches are wide, the pattern is very reminiscent of Hele-Shaw patterns¹¹ and the fractal dimension is close to two. As the peptone level is decreased, the patterns become more ramified (b and c), reminiscent of patterns observed during electrochemical deposition¹¹ (b) and diffusion limited aggregation (DLA)²² simulations (c). At even lower peptone levels the patterns become denser again (d). As explained in the text, we expect this phenomenon to result from chemotaxis signalling.

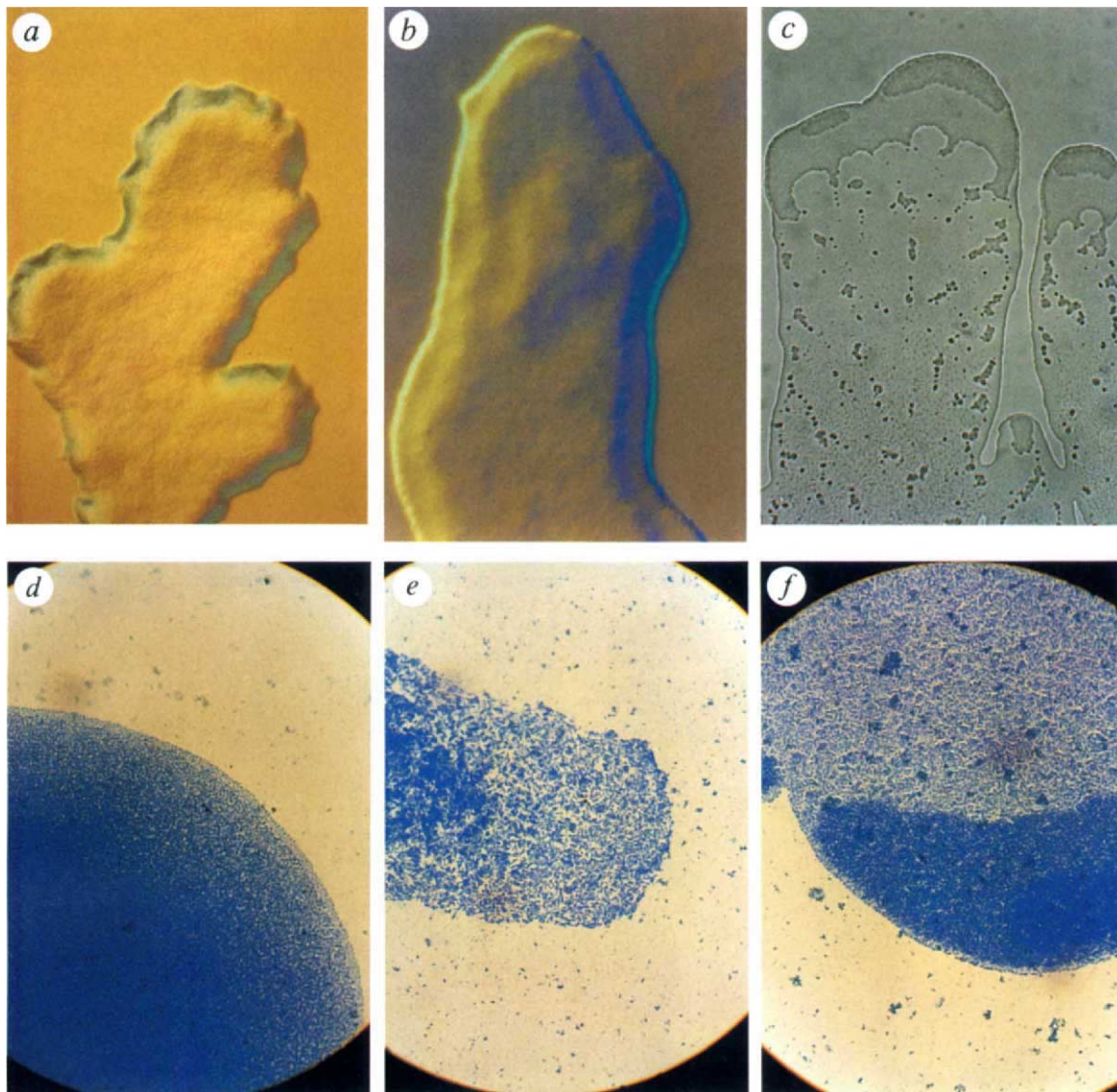


FIG. 2 Optical microscope observations of the colonies. *a* and *b*, Using a Numarski prism to indicate the sharpness of the envelopes. In *a* the envelope is rougher in the horizontal direction. *c*, Using transmitted light to show the complex three-dimensional structure of growth on substrates with a high agar concentration (2.5%). *d*, *e*, *f*, Micrographs

of stained colonies. *d*, Intermediate values of peptone level and agar concentration. *e*, Low peptone level. *f*, High agar concentration: note the higher bacteria density at the tip in this case. Magnifications: *a*, *b*, *c*, $\times 28$; *d*, *e*, *f*, $\times 280$.

diffusion equation for the nutrient concentration c on a triangular lattice. The bacteria are represented by walkers, each of which should be viewed as a mesoscopic unit (coarse graining of the colony) and not as an individual bacterium.

Each walker is described by its location $\vec{r}_i$ and an internal degree of freedom ('internal energy' W_i), which affects its activity. The walker loses 'internal energy' at a rate e . To increase the internal energy it consumes nutrients at a fixed rate c_r , if sufficient food is available. Otherwise, it consumes the available amount. When there is not enough food for an interval of time (causing W_i to drop to zero), the walker becomes stationary (sporulation). When food is sufficient, W_i increases, and when it reaches some threshold t_r , the walker divides into two (reproduction).

The walkers perform off-lattice random walk within a well defined envelope (defined on the triangular lattice in Fig. 3*a*). Each segment of the envelope moves after it has been hit N_c times by the walkers. This requirement represents the local communication or cooperation in the behaviour of the bacteria. Note that, to a first approximation, the level of N_c represents the

agar concentration, as more 'collisions' are needed to push the envelope on a harder substrate.

The model equations are:

$$\frac{\partial c(\vec{r}, t)}{\partial t} = D_c \nabla^2 c(\vec{r}, t) - \sum_{\text{active walkers}} \delta(\vec{r} - \vec{r}_i) \min(c_r, c(\vec{r}, t)) \quad (1)$$

This is the diffusion equation for the nutrients (D_c is the diffusion constant) which includes the consumption of food by the walkers (last term). The time evolution of W_i is given by:

$$\frac{dW_i}{dt} = \min(c_r, c(\vec{r}_i, t)) - e \quad (2)$$

At each time step, each of the active random walkers performs a random walk of step size d at an angle Θ , uniformly chosen from the interval $[0, 2\pi]$. Thus the new location $\vec{r}'_i$ is given by:

$$\vec{r}'_i = \vec{r}_i + d(\cos \Theta, \sin \Theta) \quad (3)$$

If the step $\vec{r}_i \rightarrow \vec{r}'_i$ crosses the envelope, the step is not performed and a counter on the appropriate segment of the envelope is

increased by one. When a segment counter reaches N_c , the envelope segment is shifted by one lattice step.

Results of numerical simulations of the model are shown in Fig. 3b. As in the growth of bacterial colonies, the patterns are compact at high peptone levels and become fractal with decreasing food level. For a given peptone level, the patterns are more ramified as the agar concentration increases. Clearly, the results shown in Fig. 3 are very encouraging and do capture features of the observed patterns. However, there are some crucial quali-

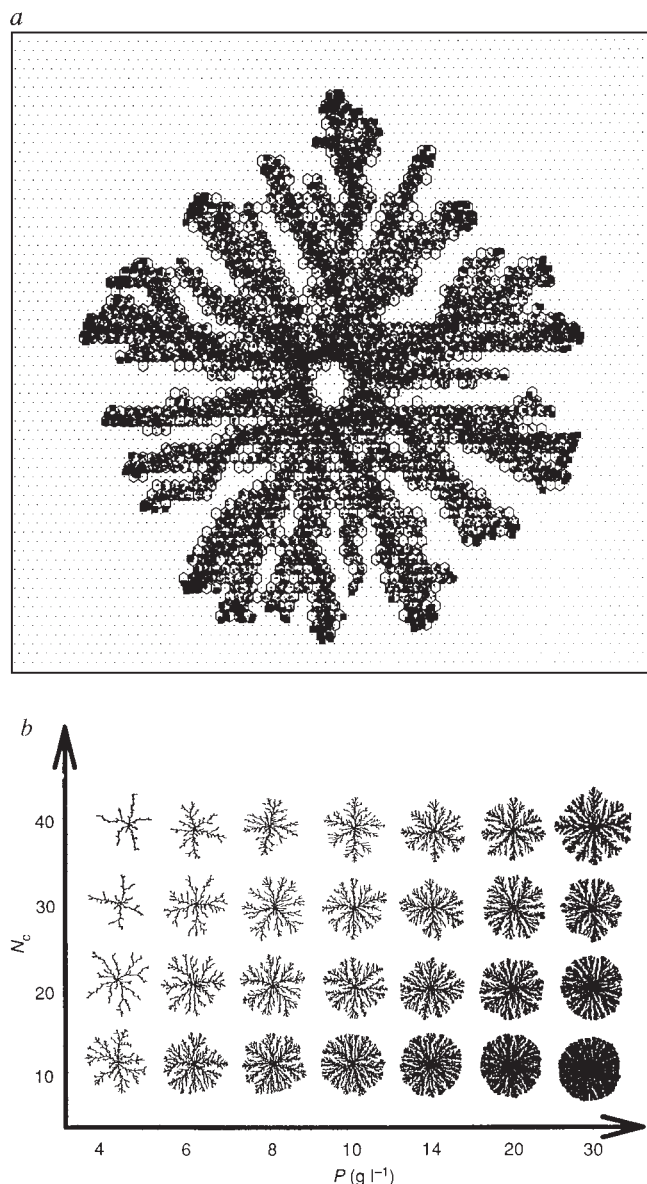


FIG. 3 The communicating walkers model. *a*, Close-up view of the model. The solid squares are the active walkers and the heavy dots are the stationary ones. Equation (1) is solved on the underlying triangular lattice. The hexagons mark the envelope. *b*, Results of numerical simulations of the communicating walkers model. The patterns are organized as function of peptone level P (the initial value of c) and N_c (corresponds to the agar concentration). The typical system size is 600×600 and the typical number of the walkers is 10^5 . Hence each walker represents $\sim 10^4$ bacteria. The observed patterns are compact for high peptone levels and become ramified for low peptone levels. For the same peptone level, the patterns are more branched for higher N_c . Both are consistent with experimental observations. Note that the fractal dimension becomes much smaller than the DLA fractal dimension²². It reflects the fact that the envelope propagation has non-trivial dependence on the gradient of the nutrients diffusion field.

tative differences. The most dramatic one is the ability of the bacteria to develop organized patterns at very low peptone levels (Fig. 1d), a feature which is not captured by this version of the model.

As the environmental conditions become more hostile (low peptone concentration or hard surface), a higher level of cooperation is required for a more efficient response of the colony. Non-local communication and transfer of information between each of the individuals and the colony might be needed. Can chemotactic signalling provide the colony with the means to do so? Generally, chemotaxis means movement of the microorganisms in response to a concentration gradient of certain chemicals⁶⁻¹⁰. Ordinarily, the movement is along the gradient, either in the direction of the gradient or the opposite direction. The chemotactic response may be to an external chemical field or to one produced by the microorganisms; the latter may be called chemotactic signalling or communication. Moreover, it is well known⁴ that excretion of the signal can be triggered by an external stress. Such non-local communication enables each bacterium to obtain information about the state of the colony as a whole and to respond to it. For example, the migration of *Dictyostelium* under low nutrient conditions depends on chemotactic signalling via cyclic adenosine monophosphate (cAMP)²¹. In this case, each of the microorganisms may excrete and consume cAMP and move according to its concentration gradient.

Here we include a simple version of chemotactic communication in the hope of identifying the generic features that it induces. Each of the stationary walkers (or alternatively, walkers which have been exposed to a low level of food) produces a communication chemical at a fixed rate s_r (in an attempt to drive other bacteria away), and each of the active walkers consumes the chemical at a fixed rate c_c . As we show below, this simplified version is sufficient to capture the qualitative features of the growth. A more realistic model would include a dependence of these rates on the concentrations of nutrients and chemotactic signalling compounds. The equation of the communication field in the present model is given by:

$$\frac{\partial s(\vec{r}, t)}{\partial t} = D_s \nabla^2 s(\vec{r}, t) + \sum_{\text{stationary walkers}} \delta(\vec{r} - \vec{r}_i) s_r - \sum_{\text{active walkers}} \delta(\vec{r} - \vec{r}_i) \min(c_c, s(\vec{r}, t)) \quad (4)$$

The movement of the active bacteria changes from pure random walk (equal probability to move along any direction) to a random walk with a bias along the gradient of the communication field (high probability to move in the direction of the signalling material).

In Fig. 4 we show that the inclusion of such chemotaxis signalling indeed produces the desired phenomena. The pattern

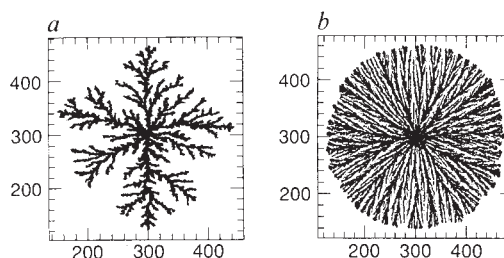


FIG. 4 Effect of chemotaxis signalling on the communicating walkers model. *a*, In the absence of the chemotaxis for $P=10 \text{ g l}^{-1}$ and $N_c=40$. *b*, In the presence of chemotaxis (for the same values of P and N_c). The pattern becomes denser with radial thin branches and well defined circular envelope, in agreement with experimental observations. The numbers on the axes represent the trigonal lattice sites to indicate the system size.

changes from fractal to a dense structure with thin branches and a well defined circular envelope. Moreover, the number of walkers (bacteria density) in the colony is much lower than in the absence of chemotaxis. We find that a chemotactic response to gradients of nutrient concentration does not reproduce this effect. Here we have simply introduced chemotactic signalling when the peptone level becomes low; in reality, there might be additional control mechanisms which change the intensity of the chemotaxis communication as the bacteria go through phenotypic transformations. We suspect that the observed perpendicular growth at high agar concentration also results from chemotactic signalling.

Our goal in this work is to demonstrate that apparently complex behaviour in biological systems can be elucidated by relatively simple, generic modelling in conjunction with a close comparison to experimental observations. □

Received 19 August; accepted 21 December 1993.

1. Stainer, R. Y., Doudoroff, M. & Adelberg, E. A. *The Microbial World* (Prentice-Hall, New Jersey, 1957).

2. Shapiro, J. A. *Scientific American* **258** (6) 62–69 (1988).
3. Shapiro, J. A. & Trubatch, D. *Physica D* **49**, 214–223 (1991).
4. Budrene, E. O. & Berg, H. C. *Nature* **349**, 630–633 (1991).
5. Kessler, J. O. *Contemp. Phys.* **26**, 147–166 (1985).
6. Adler, J. *Science* **166**, 1588–1597 (1969).
7. Lackie, J. M. (ed.) *Biology of the Chemotactic Response* (Cambridge Univ. Press, 1981).
8. Devreotes, P. *Science* **245**, 1054–1058 (1989).
9. Berg, H. C. & Purcell, E. M. *Biophys. J.* **20**, 193–219 (1977).
10. Nossal, R. *Expl. Cell Res.* **75**, 138–142 (1972).
11. Ben-Jacob, E. & Garik, P. *Nature* **343**, 523–530 (1990).
12. Ben-Jacob, E., Shmueli, H., Shochet, O. & Tenenbaum, A. *Physica A* **187**, 378–424 (1992).
13. Ben-Jacob, E., Tenenbaum, A., Shochet, O. & Avidan, O. *Physica A* **202**, 1–47 (1994).
14. Henrici, T. H. *The Biology of Bacteria* 3rd edn (Heath, Boston, 1948).
15. Cooper, A. L. *Proc. R. Soc. B* **171**, 175–199 (1968).
16. Fujikawa, H. & Matsushita, M. *J. phys. Soc. Japan* **58**, 3875–3878 (1989).
17. Matsuyama, T. & Matsushita, M. *Crit. Rev. Bact.* **19**, 117–135 (1993).
18. Schindler, J. & Rataj, T. *Binary* **4**, 66–72 (1992).
19. Allison, C. & Hughes, C. *Sci. Prog.* **75**, 403–422 (1991).
20. Henriksen, J. *Bacter. Rev.* **36**, 478–503 (1972).
21. Kessler, D. & Levine, H. *Phys. Rev. E* (in the press).
22. Sander, L. M. *Nature* **322**, 789–795 (1986).

ACKNOWLEDGEMENTS. We thank I. Brainin, D. Weiss and H. Leibovich for technical assistance. We have benefited from conversations with J. Shapiro, E. Ron, D. Gutnik, D. Kessler, H. Levine and P. Garik. This research was supported in part by the German-Israeli Foundation for Scientific Research and Development, the Hungarian Research Foundation and the Program for Alternative Thinking at Tel-Aviv University.

Productivity of pre-vascular continental biota inferred from the $\text{Fe}(\text{CO}_3)\text{OH}$ content of goethite

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CHEMICAL weathering of rocks by CO_2 —an important process in the carbon cycle—may have been affected by the appearance of vascular plants during the Silurian period (438–408 Myr ago), because of the enhanced efficacy of these plants in promoting weathering¹. The magnitude of this effect is uncertain, however^{2–4}. One key issue is the concentration of CO_2 in soils, generated by microbial respiration; this is itself in part a function of soil productivity. We showed recently⁵ that the CO_2 partial pressure in a Late Ordovician soil was 5.6 times that in the atmosphere; Keller and Wood⁶ have shown, however, that soil CO_2 concentrations can be high even for low levels of microbial respiration, as a result of slow diffusion of respired CO_2 out of the soil. Here we present an analysis of the $\text{Fe}(\text{CO}_3)\text{OH}$ component of goethite (FeOOH) in an Upper Ordovician oolitic ironstone which underwent tropical chemical weathering 440 Myr ago^{7,8}. These data allow us to extract an estimate of the CO_2 flux from the soil

profile. The high rate that we obtain implies that the productivity of pre-vascular biota was similar to that in modern soils. Thus, attempts to model atmospheric CO_2 concentrations over Phanerozoic^{9–12} time need not assume that pronounced productivity changes accompanied the development of vascular plants.

The common mineral goethite ($\alpha\text{-FeOOH}$) contains an $\text{Fe}(\text{CO}_3)\text{OH}$ component in apparent solid solution^{13–15}. The abundance and $^{13}\text{C}/^{12}\text{C}$ ratio of this component seem to be a measure of the partial pressure (p_{CO_2}) and carbon isotope ratio, respectively, of CO_2 present at the time of goethite crystallization. The Upper Ordovician Neda Formation at its type locality near Neda, Wisconsin, USA, is a goethite-dominated, oolitic ironstone whose upper surface is a Late Ordovician unconformity⁷. The goethite in this ironstone has apparently remained a closed system since the time of its formation in the Late Ordovician ~440 Myr ago^{5,8,16}. A subaerial weathering origin for this ancient goethite at a temperature of ~23 °C is supported by the existence of the unconformity⁷, by oxygen isotope data which indicate the presence of meteoric water ($\delta^{18}\text{O} = -7.3$) at the time of crystallization⁸, and by the vertical distribution of the carbon isotope and mole fraction data of the $\text{Fe}(\text{CO}_3)\text{OH}$ component¹⁷. The measured mole fractions (X) of the $\text{Fe}(\text{CO}_3)\text{OH}$ component in different Neda Formation goethite samples are plotted as a function of sample depth below the unconformity in Fig. 1a. These data are tabulated in ref. 5. Values of X increase relatively rapidly from the unconformity to a depth of ~30 cm. The changes in X are more gradual at greater depths. If the changes in X in Fig. 1a reflect variations in ancient ambient p_{CO_2} , the indicated pattern is generally con-

FIG. 1 a, b, Mole fractions (X) of the $\text{Fe}(\text{CO}_3)\text{OH}$ component in goethite of the Neda Formation oolitic ironstone plotted as a function of sample depth (z) below the unconformity that defines the top of the deposit (the dashed line at $z=0$). The width of each data symbol represents the analytical uncertainty of ± 0.0002 for values of X . The height of each symbol corresponds to the vertical interval represented by the sample. These data are tabulated in ref. 5. The curved lines represent model calculations for one-dimensional steady-state, diffusive transport of CO_2 in a soil profile (see text). The calculated model curve in a assumes that the existing unconformity corresponds to the original weathering surface (no offset). The curve in b was calculated assuming that 20 cm of the deposit were eroded ($z+20$) before the deposit was buried beneath Early Silurian marine carbonates (see text).

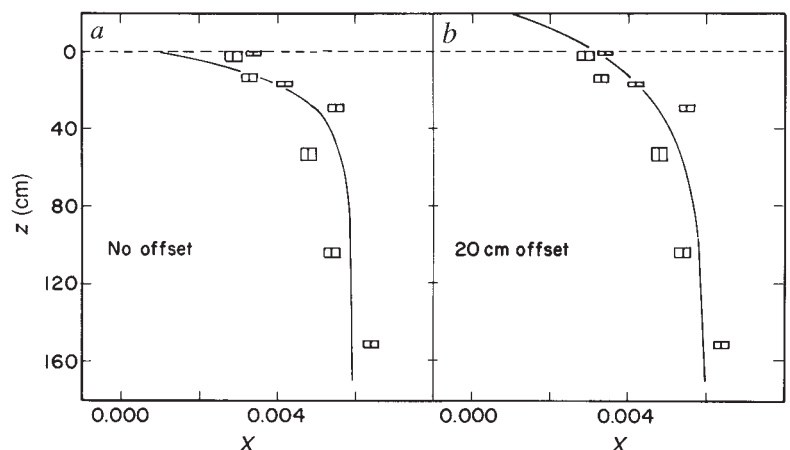
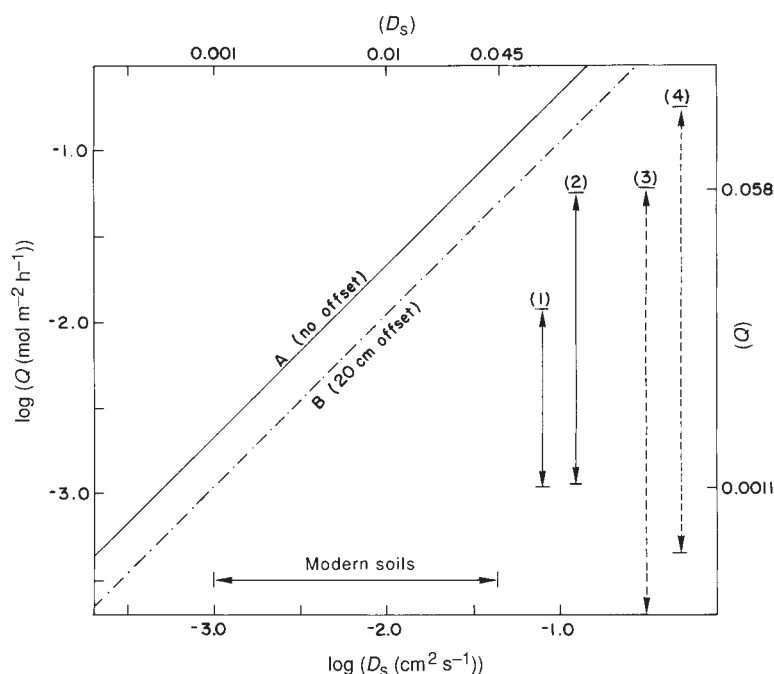


FIG. 2 Log-log plot of the calculated vertical CO₂ flux (Q) in mol m⁻² h⁻¹ out of the Neda Formation weathering profile as a function of the presumed soil CO₂ diffusion coefficient (D_s) in cm² s⁻¹. These curves were calculated for the model curves of Fig. 1a, b. Note that the assumption that some of the original deposit was eroded (20 cm offset) leads to a lower calculated value of Q at all values of D_s (line B). For reference, vertical arrows indicate the ranges of Q values measured for various modern soil environments²⁵; (1) represents tropical savannas and grasslands; (2) tropical forests; (3) temperate prairies and grasslands; and (4) temperate forests. Also shown is an approximate range (0.001–0.045) for D_s values in the upper 2 m of different soils as calculated from the water content and porosity values of Keller and Wood⁶ and results reported in Jury *et al.*³². The range of Q values on the right-hand axis indicates the range measured in modern soils²⁵. The solid vertical arrows represent tropical soils, and the dashed arrows represent temperate soils.



sistent with that of modern soils, in which soil CO₂ undergoes steady-state diffusive transport to the Earth's atmosphere^{18–20}.

Yapp and Poths⁵ determined that the ancient p_{CO_2} in the Neda Formation weathering profile was ~5.6 times greater than the value in the atmosphere during the Late Ordovician period. They also determined that the Earth's atmospheric p_{CO_2} at that time was ~16 or 17 times greater than the modern atmospheric p_{CO_2} (refs 5, 17). Therefore, the p_{CO_2} in the ancient 'soil' might have been ~90 times greater than the value in the Earth's modern atmosphere. These quantitative estimates of ancient p_{CO_2} values are independent of any assumed values for soil CO₂ respiration. In modern soils higher values of p_{CO_2} at depth arise from *in situ* oxidation of organic matter^{18,21,22}. Yapp and Poths calculated a $\delta^{13}\text{C}$ value of ~-27‰ for the carbon that was the source of CO₂ produced *in situ* during Late Ordovician weathering of the Neda Formation ironstone⁵. This $\delta^{13}\text{C}$ value is consistent with oxidation of biologically-derived carbon 440 Myr ago. Therefore, steady-state fickian diffusive transport with a depth-dependent CO₂ production term will be employed to represent the Neda Formation CO₂ data. It is assumed that the *in situ* production of CO₂ declined exponentially with depth (see refs 5, 22, 23) and that the diffusion coefficient (D_s) of CO₂ in the ancient weathering profile was constant.

The following steady-state solution to the one-dimensional diffusion equation was obtained for Fe(CO₃)OH as an indicator of ancient soil CO₂:

$$X_s = \frac{K\bar{z}^2\phi_0}{D_s} [1 - e^{-z/\bar{z}}] + X_A \quad (1)$$

where z is the depth (cm) below the surface of the profile, and is positive below the unconformity, $\bar{z}$ is the characteristic (or scaling) depth, ϕ_0 is the *in situ* production of CO₂ at $z=0$ (moles per unit volume per unit time), $K=RT(X/p_{\text{CO}_2})$ R is the gas constant, T is the thermodynamic temperature and X/p_{CO_2} is the Henry's Law constant for equilibrium between Fe(CO₃)OH and gaseous CO₂ at the time of goethite crystallization. X_s is the measured mole fraction of Fe(CO₃)OH in a goethite sample at some depth z and X_A would be the mole fraction of Fe(CO₃)OH if it were in equilibrium only with ancient atmospheric CO₂ (an X_A value of 0.000975 was determined by Yapp and Poths for the Late Ordovician⁵). The boundary conditions implicit in equation (1) are: $X_s = X_A$ at $z=0$, and $\partial X_s/\partial z \rightarrow 0$ as $z \rightarrow \infty$.

The calculated model curve in Fig. 1a is represented by the following equation:

$$X_s = 0.004929 (1 - e^{-z/18.2}) + 0.000975 \quad (2)$$

Equation (2) assumes that the surface of the existing unconformity corresponds to the original weathering surface of the deposit. This assumption would be incorrect, if, for example, an upper portion of the ironstone was eroded when the sea covered the deposit in the Early Silurian marine transgression⁷. We do not know how much, if any, of the original profile was eroded during the marine transgression, but as an illustration of the effect of such erosion on the calculated diffusive transport curve, we have assumed that 20 cm of the original weathering profile were removed. It seems unlikely that much more of the deposit could have been eroded and still preserve a record of a p_{CO_2} profile which has the previously mentioned characteristics of a 'soil' at the shallower depths^{18–20}. The calculated curve for this 20 cm 'offset' in the z coordinate is shown in Fig. 1b. The curve of Fig. 1b was forced to pass through the same X values at 42 cm and 148 cm in the offset coordinates ($z+20$) as those X values passed through at 22 cm (0.00443) and 128 cm (0.00590) by the curve of Fig. 1a. This facilitated direct comparison of the effect of the offset on the relationship of the model curves to the data set in the upper portion of the profile. The equation representing the curve in Fig. 1b is:

$$X_s = 0.005004 (1 - e^{-z/35.7}) + 0.000975 \quad (3)$$

The value of X_A is the same in equations (2) and (3), because the atmospheric boundary conditions for each scenario are identical. The sum of the squares of the residuals for the curve of Fig. 1a is 9.2×10^{-6} , whereas for Fig. 1b it is 1.8×10^{-6} . By this criterion, the curve of Fig. 1b is a better representation of the data set than is the curve of Fig. 1a. The largest discrepancies between the calculated curve and the measured data in Fig. 1a are for the two shallowest samples, which were collected immediately at the unconformity⁵. If they were actually at the original weathering surface when they crystallized, these shallow samples would be expected to have X values closer to that of X_A . The fact that both have higher values of X suggests that at least some portion of the deposit was eroded before burial beneath the Early Silurian marine carbonate muds.

Although there is uncertainty concerning the position of the

original weathering surface, as well as some scatter in the data about the curves, the general correspondence between the data set and the calculated model curves of Fig. 1 suggests that model-dependent estimates of an additional palaeoenvironmental parameter could be worthwhile. The vertical flux of CO_2 out of the weathering profile is termed Q , where $Q = \bar{z}\phi_0$ (ref. 20). Examination of equation (1) suggests that steady-state values of the ratio Q/D_s can be calculated from equations (2) and (3) if the value of K is known. A value of K at 23°C ($4,491\text{ cm}^3\text{ mol}^{-1}$) was calculated using the Henry's Law equation of Yapp and Poths¹⁷. Calculated curves in Fig. 2 depict the relationship between Q and D_s for the model curves of Fig. 1. An uncertainty in temperature of $\pm 4^\circ\text{C}$ (ref. 8) would result in a relative uncertainty of $\sim \pm 16\%$ in the calculated value of Q/D_s as a consequence of the temperature dependence of K . Values of Q for the 'no offset' curve of Fig. 2 (curve A) are about twice as large as those for the '20 cm offset' curve (B) at all values of D_s . We discuss the curve in Fig. 2 for the case of 20 cm offset, because it represents the more conservative example.

The value of the soil CO_2 diffusion coefficient (D_s) depends on the free-air porosity and tortuosity of the soil^{18,21}. Cerling has successfully used a value of $0.02\text{ cm}^2\text{ s}^{-1}$ in model calculations pertaining to calcite-rich soils¹⁸. We have no estimates of the uncompacted porosity and tortuosity of the Neda Formation weathering profile, but we adopt a value of $\sim 0.01\text{ cm}^2\text{ s}^{-1}$ as an estimate of D_s in the upper 2 m, because iron-oxide-rich weathering systems are generally wetter than carbonate-rich soils²⁴. Thus, free-air porosity at the time of goethite crystallization might have been lower, thereby producing a lower value of D_s . For the case of 20 cm offset and the selected value of D_s , the value of Q from Fig. 2 is $\sim 0.01\text{ mol m}^{-2}\text{ h}^{-1}$. This value lies within the range of Q values measured in modern tropical and temperate soils²⁵. Keller and Wood⁶ have modelled soil p_{CO_2} for a specified soil respiration rate using a constant *in situ* CO_2 production rate and an effective CO_2 diffusion coefficient that decreases from the surface to a depth of 10 m. None of their calculated model soil p_{CO_2} values are as large at depths of $\sim 2\text{ m}$ as the p_{CO_2} determined for the Neda Formation at such depths. Our higher soil p_{CO_2} values could suggest higher CO_2 fluxes. But as shown in Fig. 2, the approximate range of D_s values in the upper 2 m of different modern soils is large. Therefore, the higher soil p_{CO_2} of the Neda Formation would also be consistent with fluxes $< 0.01\text{ mol m}^{-2}\text{ h}^{-1}$ for D_s values $< 0.01\text{ cm}^2\text{ s}^{-1}$. Yet even if D_s values in the Neda Formation were as small as $0.001\text{ cm}^2\text{ s}^{-1}$ the value of Q for this ancient tropical system would lie well within the range of modern temperate soils (see Fig. 2).

Although this deposit was probably buried to depths of $< 0.5\text{ km}$ (ref. 26), it should be noted that burial compaction would have reduced the original vertical separation between samples in the weathering profile. Thus, the values of z shown in Fig. 1 are probably underestimates of the original, uncompacted values, even if erosion were correctly taken into account. This 'compression' of z values would produce an erroneously high estimate of the CO_2 flux. As an illustration of the sensitivity of Q to compaction, the foreshortening expected for burial to a depth of 1 km was calculated. No compaction curve for oolitic ironstone is available, but a sandstone curve was used as a first approximation²⁷. The calculated reduction in thickness of the deposit is $\sim 18.6\%$. For this degree of thinning, the calculated CO_2 flux is $\sim 23\%$ higher without correction for compaction, than it would have been with such correction. The uncertainties arising from compaction and the effect of temperature on K combine to produce a possible overestimate of Q of $\sim 46\%$.

The Neda Formation ironstone was exposed to subaerial weathering when sea level fell at the end of the Ordovician period, 440 Myr ago⁷. This drop in sea level was probably global and a result of growth of the continental ice sheet on Gondwanaland²⁸. Some evidence from marine fossils in Europe suggests that this drop in sea level may have had a duration of up to 2 Myr (ref. 28). Therefore, the opportunity for subaerial

chemical weathering of the Neda Formation ironstone might have existed for that length of time. To our knowledge, the work reported here is the first attempt to derive a quantitative estimate of ancient soil CO_2 respiration rates from a measured indicator of p_{CO_2} . The estimate is for a highly evolved, iron oxide-rich, chemical weathering system. The chemical composition of this system suggests that it was a relatively nutrient-poor environment^{6,16}. If the pre-vascular terrestrial biological activity deduced from our data was somehow sustained for up to 2 Myr, it implies a mechanism for recycling nutrients within the weathering profile, an aeolian source, or both. Furthermore, if our inference about the magnitude of the CO_2 flux is approximately correct, it indicates the presence on that tropical continental site of an ancient pre-vascular terrestrial biota as productive as biota of modern tropical and/or temperate soils. Such biota may have played a role in the chemical weathering of the continents before the widespread colonization by vascular plants²⁹⁻³¹. □

Received 20 July 1993; accepted 11 January 1994.

1. Berner, R. A. *Geochim. cosmochim. Acta* **56**, 3225-3231 (1992).
2. Jackson, T. A. *Geochim. cosmochim. Acta* **57**, 2141-2144 (1993).
3. Schwartzman, D. *Geochim. cosmochim. Acta* **57**, 2145-2146 (1993).
4. Cochran, M. F. & Berner, R. A. *Geochim. cosmochim. Acta* **57**, 2147-2148 (1993).
5. Yapp, C. J. & Poths, H. *Geochim. cosmochim. Acta* **57**, 2599-2611 (1993).
6. Keller, C. K. & Wood, B. D. *Nature* **364**, 223-225 (1993).
7. Paull, R. A. *Geology of Southeastern Wisconsin 41st Tri-State Field Conf.* (ed. Nelson, K. G.) C1-C18 (Univ. Wisconsin, Milwaukee, 1977).
8. Yapp, C. J. *Geochim. cosmochim. Acta* **57**, 2319-2327 (1993).
9. Volk, T. *Am. J. Sci.* **287**, 763-779 (1987).
10. Berner, R. A. *Science* **249**, 1382-1386 (1990).
11. Berner, R. A. *Am. J. Sci.* **291**, 339-376 (1991).
12. Francois, L. M. & Walker, J. C. G. *Am. J. Sci.* **292**, 81-135 (1992).
13. Yapp, C. J. *Chem. Geol.* **64**, 259-268 (1987).
14. Yapp, C. J. & Poths, H. *Clays clay Miner.* **38**, 442-444 (1990).
15. Yapp, C. J. & Poths, H. in *Stable Isotope Geochemistry: A Tribute to Samuel Epstein* (eds Taylor, H. P. Jr., O'Neill, J. R. & Kaplan, I. R.) 257-270 (Geochem. Soc. Spec. Publ. No. 3, Geochem. Soc., San Antonio, 1991).
16. Yapp, C. J. *Geochim. cosmochim. Acta* **55**, 2627-2634 (1991).
17. Yapp, C. J. & Poths, H. *Nature* **355**, 342-344 (1992).
18. Cerling, T. E. *Earth planet. Sci. Lett.* **71**, 229-240 (1984).
19. Quade, J., Cerling, T. E. & Bowman, J. R. *Geol. Soc. Am. Bull.* **101**, 464-475 (1989).
20. Solomon, D. K. & Cerling, T. E. *Water Resour. Res.* **23**, 2257-2265 (1987).
21. Hillel, D. *Introduction to Soil Physics* (Academic, San Diego, 1982).
22. Wood, W. W. & Petraitis, M. J. *Water Resour. Res.* **20**, 1193-1208 (1984).
23. Cerling, T. E. *Am. J. Sci.* **291**, 377-400 (1991).
24. Tardy, Y., Kobalsek, B., Roquin, C. & Paguet, H. *Chem. Geol.* **84**, 179-182 (1990).
25. Singh, J. S. & Gupta, S. R. *Bot. Rev.* **43**, 449-528 (1977).
26. Smith, G. L. *Geol. Soc. Am. Abstr. Progr. No. 7* **22**, A89 (1990).
27. Baldwin, B. & Butler, C. O. *Bull. Am. Ass. Petrol. Geol.* **69**, 622-626 (1985).
28. Hambrey, M. J. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **51**, 273-289 (1985).
29. Gensel, P. G. & Andrews, H. N. *Am. Sci.* **75**, 478-489 (1987).
30. Retallack, G. & Feakes, C. *Science* **235**, 61-63 (1987).
31. Gray, J. & Shear, W. *Am. Sci.* **80**, 444-456 (1992).
32. Jury, W. A., Gardner, W. R. & Gardner, W. H. *Soil Physics* 5th edn (Wiley, New York, 1991).

ACKNOWLEDGEMENTS. We thank G. Retallack for comments on the manuscript. This work was supported by the US National Science Foundation.

Geomagnetic field morphologies from a kinematic dynamo model

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THE Earth's magnetic field is generated by flow of liquid iron in the outer core, acting as a dynamo. In the simple kinematic theory a fluid flow is prescribed and tested for its ability to generate magnetic field, no account being taken of the forces required to drive the flow. Although incomplete, kinematic theory gives valuable insight into the more difficult dynamical problem and produces field morphologies which can be compared with observations. We are attempting a comprehensive study of three-dimensional kinematic dynamo action for a class of fluid flow typical of that driven by convection (G.S. and D.G., manuscript in preparation), and

have already found similarities between steady dipole solutions and the geomagnetic field¹. Here we examine the effect of meridian circulation in determining the stability of steady and oscillatory solutions. The magnetic field morphology on both sides of the stability boundary is determined by magnetic flux concentration by downwelling fluid². The oscillatory dipole reverses polarity through the appearance and poleward migration of patches of reversed flux, similar to those seen in today's geomagnetic field³. Virtual geomagnetic poles computed from the reversing field follow paths that are concentrated along the longitudes of maximum flux, suggesting a link with recent palaeomagnetic results⁴⁻⁷.

The magnetic field $\mathbf{B}$ obeys the induction equation

$$\frac{\partial \mathbf{B}}{\partial t} = R_m \nabla \times (\mathbf{v} \times \mathbf{B}) + \nabla^2 \mathbf{B} \quad (1)$$

where $\mathbf{v}$ is the fluid velocity, $R_m = \mathcal{U} c \mu_0 \sigma$ the magnetic Reynolds number, $\mathcal{U}$ the velocity scale, c the linear dimension (core radius for the Earth), σ the electrical conductivity and μ_0 the magnetic permeability of free space. $\mathbf{v}$ is prescribed, so that equation (1) is linear in $\mathbf{B}$ and has solutions that vary in time, t , as $\exp(p + i2\pi f)t$. Dynamo action occurs if we can find a critical value of R_m , R_m^c , such that the growth rate $p \geq 0$ ($\mathbf{B}$ does not decay with time). At marginal stability $p = 0$ and if $f = 0$ the solution is steady, otherwise it is oscillatory with frequency f .

Although this kinematic approach cannot reproduce complicated time variations such as irregular polarity reversals, it can increase our understanding of the physical processes occurring in dynamo action at a time when full simulations of the dynamical behaviour of a model of the Earth's core are not feasible. For example, the Parker-Levy⁸⁻¹⁰ reversal mechanism is based implicitly on kinematic results.

Despite its mathematical simplicity, the kinematic dynamo problem poses severe numerical difficulties: apparent 'solutions'^{11,12} have subsequently been shown¹³⁻¹⁵ to be numerical artefacts. Somewhat greater success was achieved with α -effect dynamos, which in the geophysically relevant Braginsky¹⁶ formulation involves averaging in longitude and taking the limit $R_m \gg 1$ to yield equations for the axisymmetric field $\bar{\mathbf{B}}$:

$$\begin{aligned} \frac{\partial \bar{\mathbf{B}}}{\partial t} = R_m \{ \nabla \times (\omega \hat{\phi} \times \bar{\mathbf{B}}_p) + \nabla \times [(\bar{\mathbf{v}} + \bar{\mathbf{v}}_{ep}) \times \bar{\mathbf{B}}] \\ + \nabla \times (\alpha \bar{\mathbf{B}}_{\phi\phi}) \} + \nabla^2 \bar{\mathbf{B}} \end{aligned} \quad (2)$$

where subscript p denotes the meridian component, $\hat{\phi}$ is a unit azimuthal vector, ω is the differential rotation, overbars denote axisymmetric quantities, α is related to the helicity $\mathbf{v} \cdot \nabla \times \mathbf{v}$, and $\bar{\mathbf{v}}_{ep}$ is an 'effective meridian circulation' which, like α , is derived from products of components of the original flow. Successful numerical solutions to the simpler $\alpha\omega$ equation (2) have been extensively documented^{17,18}, including some dynamical studies¹⁹⁻²², but they cannot produce non-axisymmetric morphology for comparison with the geomagnetic field.

We have therefore conducted a series of calculations on three-dimensional (3D) kinematic dynamos using the numerical method of Bullard and Gellman¹¹ as implemented by Gubbins¹⁴, but carried to higher numerical accuracy than previously. Three-dimensional calculations and Braginsky limit calculations have been carried out in parallel; correspondence between the two systems allows us to have confidence in the accuracy of our solutions.

Our basic fluid flow is that chosen by Kumar and Roberts²³, with the general form:

$$\mathbf{v} = t_1^0 \hat{\mathbf{e}}_1 + \varepsilon_1 s_2^0 + \varepsilon_2 s_2^{2s} + \varepsilon_3 s_2^{2c}$$

where

$$\begin{aligned} t_i^{mc} &= \nabla \times [t_i^{mc}(r) P_i^m(\cos \theta) \cos m\phi \hat{\mathbf{r}}] \\ s_i^{mc} &= \nabla \times \nabla \times [s_i^{mc}(r) P_i^m(\cos \theta) \cos m\phi \hat{\mathbf{r}}] \end{aligned}$$

Here $P_i^m(\cos \theta)$ is an associated Legendre function, $\hat{\mathbf{r}}$ a unit

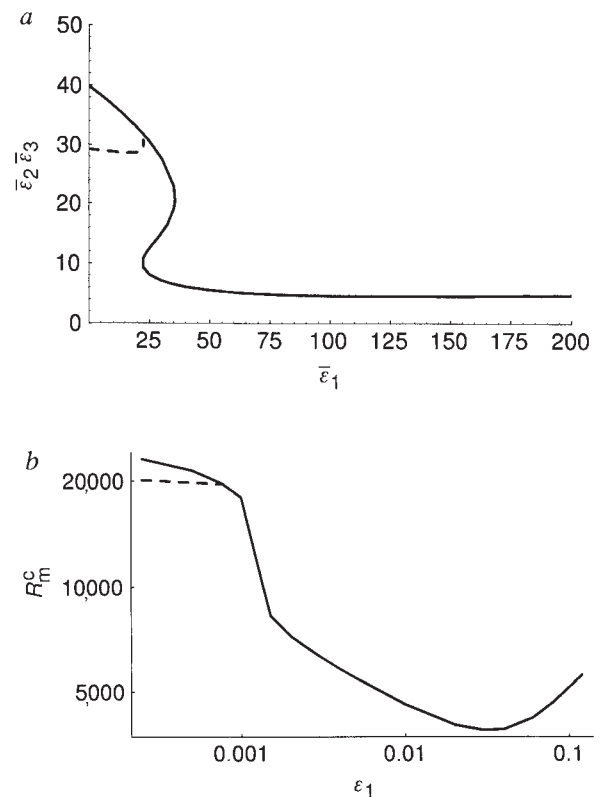


FIG. 1 Marginal stability curves as a function of meridian circulation. The lines give the critical dynamo parameters (see text for definitions of ε_1 , $\bar{\varepsilon}_1$, $\bar{\varepsilon}_2$, $\bar{\varepsilon}_3$ and R_m^c) (solution neither grows nor decays): solid (dashed) lines denote steady (oscillatory) solutions. a, Braginsky limit $\alpha\omega$ system. b, 3D system, $\varepsilon_2 = \varepsilon_3 = 0.04$. When two solutions exist for one value of ε_1 ($\bar{\varepsilon}_1$), the one with smaller R_m^c ($\bar{\varepsilon}_2, \bar{\varepsilon}_3$) is physically preferred.

radial vector, and the second superscript denotes cos or sin dependence on ϕ . The t_1^0 harmonic provides differential rotation (the ω -effect), the s_2^0 meridian circulation, and the s_2^{2s} harmonics overturning. R_m is scaled with the differential rotation; the parameters ε_i give the relative importance of other harmonics. The case $\varepsilon_1 = 0$ corresponds to Lilley's¹² dynamo with a different radial dependence prescribed. The radial dependence used here is: $t_1^0(r) = r^2(1-r^2)$, $s_2^0(r) = r^6(1-r^2)^3$, $s_2^{2s}(r) = r^4(1-r^2)^2 \cos(nr)$, $s_2^{2c}(r) = r^4(1-r^2)^2 \sin(nr)$; we illustrate our results with $n = 3\pi$, corresponding to convection with three cells in radius. Comparable results to those presented here have been obtained for different values on n (G.S. and D.G. manuscript in preparation).

The Braginsky asymptotic limit has, in this case, the complicated form: $\varepsilon_1 \rightarrow 0$, $R_m \rightarrow \infty$, $\bar{\varepsilon}_1 = \varepsilon_1 R_m = \text{constant}$, and $\varepsilon_2 \rightarrow 0$, $\varepsilon_3 \rightarrow 0$, $R_m \rightarrow \infty$, $\bar{\varepsilon}_2 \bar{\varepsilon}_3 = \varepsilon_2 \varepsilon_3 R_m = \text{constant}$. $R_m \alpha$ in equation (2) becomes replaced by $\bar{\varepsilon}_2 \bar{\varepsilon}_3$. α and $\bar{\mathbf{v}}_{ep}$ may be calculated from the original flow as described by Braginsky¹⁶ and are given for this case by Kumar and Roberts²³; note that the effective meridian circulation means equation (2) contains a $\bar{\mathbf{v}}$ even in the Lilley case $\varepsilon_1 = 0$.

Figure 1a shows marginal stability curves for the Braginsky limit case; Fig 1b gives the corresponding 3D solutions. The oscillatory solution is preferred for $\varepsilon_1 \leq 0.0008$ ($\bar{\varepsilon}_1 \leq 22.5$). Roberts¹⁷ conclusion for $\alpha\omega$ models, that meridian circulation promotes stationary solutions, is therefore confirmed in 3D, although we should strictly refer to 'effective meridian circulation' for the Braginsky limit results.

The radial component of the magnetic field at the core-mantle boundary for steady solutions exhibits flux concentrations (Fig. 2a, b) resembling those of the modern (1980) geomagnetic field

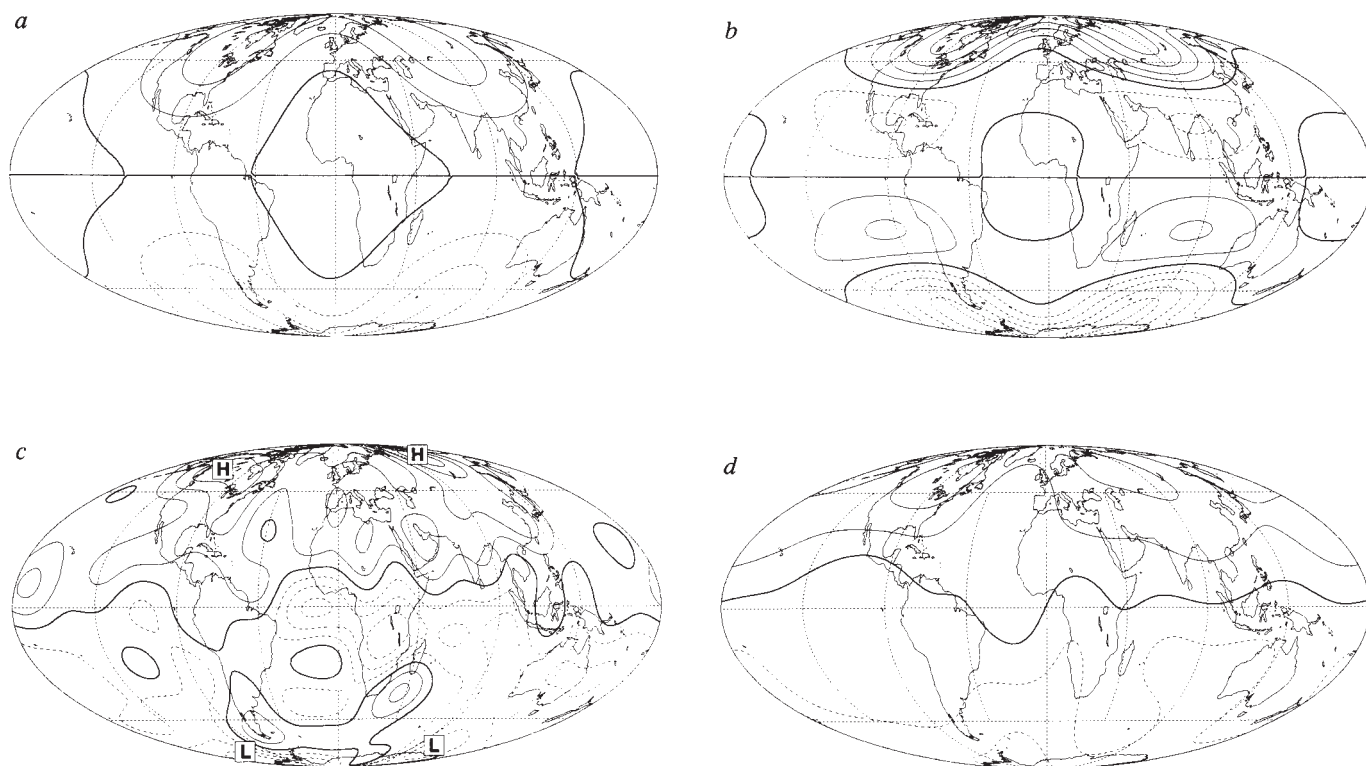


FIG. 2 Plots of the radial component of magnetic field (B_r) at the core-mantle boundary (CMB). *a*, For output of kinematic dynamo calculations for the Kumar and Roberts²³ velocity model, with $\varepsilon_1=0.03$, $\varepsilon_2=\varepsilon_3=0.04$, a case previously studied by Kumar and Roberts. Magnetic flux is concentrated by fluid downwelling approximately along longitudes 90° W and 90° E and dispersed by upwelling along 0° and 180° , as discussed by Hutcheson and Gubbins¹. *b*, For the same calculations, but using $\varepsilon_1=0.001$ and $\varepsilon_2=\varepsilon_3=0.04$, a steady solution close to where the oscillatory solution takes over. The principal difference between this and *a* is the presence of reversed flux at low latitudes. *c*, For the modern (1980) geomagnetic field²⁸, for comparison with the kinematic output. Gubbins and Bloxham²⁸ pointed out that the four main geomagnetic flux concentrations (marked H and L) are remark-

ably symmetric about the equator and positioned where convection rolls might intersect the core surface; our calculations confirm that convective downwelling could indeed produce such an effect. *d*, For the time-averaged palaeomagnetic field for the past 2.5 Myr (ref. 5), for comparison with flux concentrations of kinematic output. In *a-d*, solid (dashed) lines show concentrations of positive (negative) magnetic flux; bold lines show the zero contour. The axial dipole strengths are normalized to the present-day value of the geomagnetic dipole; contours are at $200\text{-}\mu\text{T}$ intervals. We include maps of the continents on our kinematic plots to facilitate comparison with the observed geomagnetic field; the positioning with respect to longitude is arbitrarily fixed by the definition of the velocity.

(Fig. 2c) and the time-averaged palaeomagnetic field for the past 2.5 Myr (ref. 5) (Fig. 2d). The positions of these flux patches can be related to the longitudes at which downwelling occurs in our velocity model², as might be anticipated from consideration of flux advection.

Figure 3 shows the surface field for the oscillatory solution in the Lilley case ($\varepsilon_1=0$). The morphology when the axial dipole component is strongest is very similar to that of the nearby steady solution. This solution has a period of approximately half the electromagnetic diffusion time—in geophysical terms a reversal time of $\sim 5,000$ years—and reverses by a dynamo wave mechanism¹⁰.

We can compare the transition field morphology obtained from the dynamo model with that of the geomagnetic field. First note that a large region of reversed flux has grown in the past 100 years in the South Atlantic and Indian oceans³ (Fig. 2c), suggestive of the reverse flux patches in the oscillatory solution (Fig. 3) and in nearby stationary solutions (Fig. 2b). Gubbins³ has suggested that reversals may initiate in this way. Observed Virtual Geomagnetic Pole (VGP) transition paths seem to concentrate through the Americas, with a second preferred path through Asia^{6,7,24,25}; this could be produced by migration of patches of reversed flux²⁶.

VGP transition paths are easy to calculate from the dynamo fields, and several are shown in Fig. 4; the paths tend to cluster

along the two longitudes at which the main flux lobes of the field are concentrated, as might be intuitively expected, and as is observed for the Earth²⁵. (This conclusion contrasts with that of Honkura *et al.*²⁷, who found no preferred longitude bands for VGP paths calculated from a nonlinear dynamo model. We feel, however, that this model may have numerical convergence problems, and in any case the parameter values used are far from those expected in the Earth.)

With a transition field such as that in our model, two distinct paths arise not from different reversals but from different sites; the palaeomagnetist would see two bands, with any apparent preference caused by geographical bias of the sites. That such simple VGP paths can be so site-dependent should deter palaeomagnetists from drawing conclusions regarding the morphology of transition fields from analysis of VGP data alone; we emphasize the need for a good geographical spread in any database used to seek preferred paths. Clement⁷, for example, has found two distinct paths from the same reversal recorded at different sites.

Geomagnetic reversals are not oscillations; they consist typically of one transition. Kinematic theory cannot simulate this behaviour—nonlinear calculations are required—but it might give a guide to possible nonlinear behaviour. If the dynamo were operating with a flow similar to those studied here, close to the steady-oscillatory boundary, its time variations

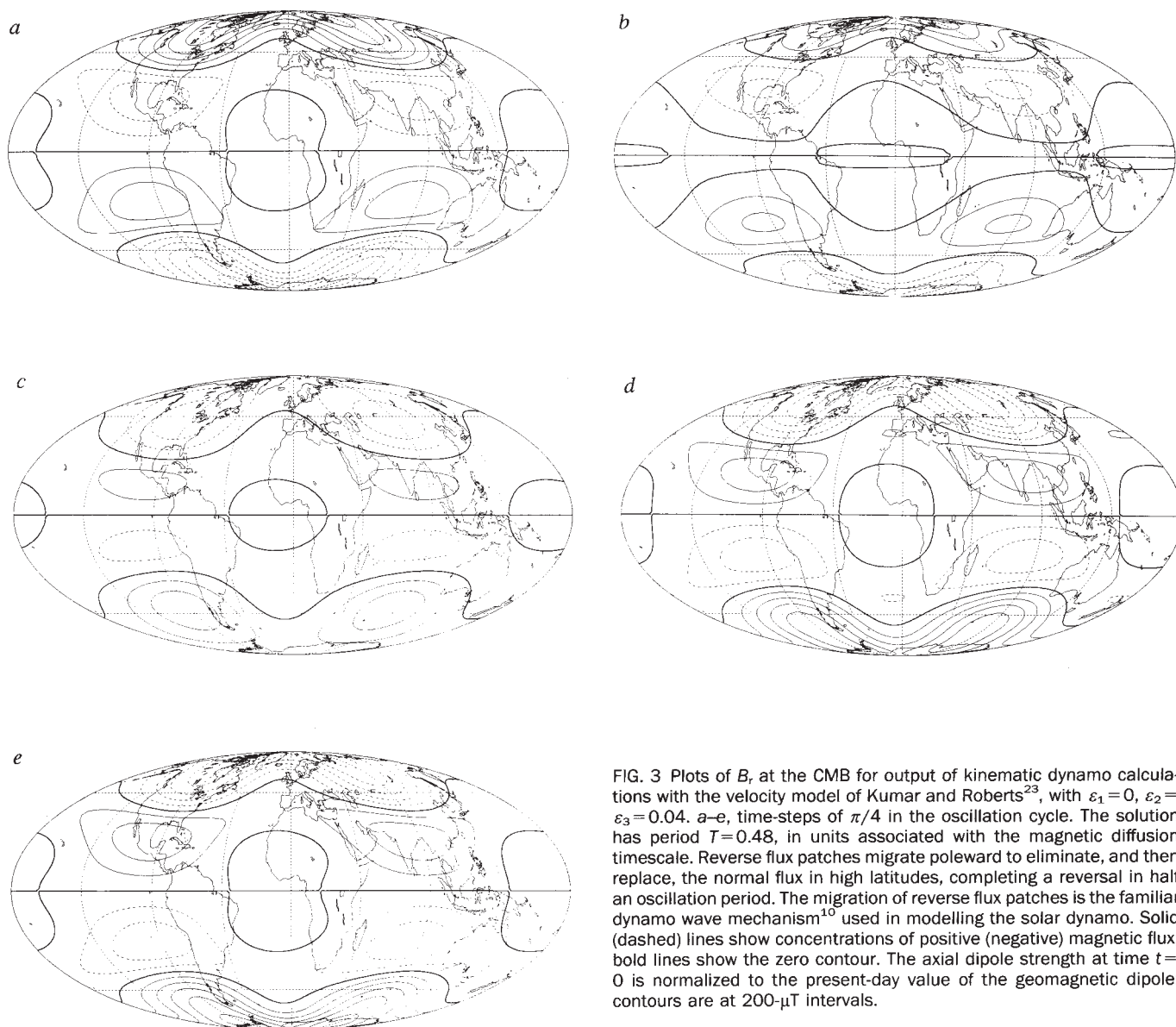


FIG. 3 Plots of B_r at the CMB for output of kinematic dynamo calculations with the velocity model of Kumar and Roberts²³, with $\varepsilon_1=0$, $\varepsilon_2=\varepsilon_3=0.04$. *a-e*, time-steps of $\pi/4$ in the oscillation cycle. The solution has period $T=0.48$, in units associated with the magnetic diffusion timescale. Reverse flux patches migrate poleward to eliminate, and then replace, the normal flux in high latitudes, completing a reversal in half an oscillation period. The migration of reverse flux patches is the familiar dynamo wave mechanism¹⁰ used in modelling the solar dynamo. Solid (dashed) lines show concentrations of positive (negative) magnetic flux; bold lines show the zero contour. The axial dipole strength at time $t=0$ is normalized to the present-day value of the geomagnetic dipole; contours are at 200- μ T intervals.

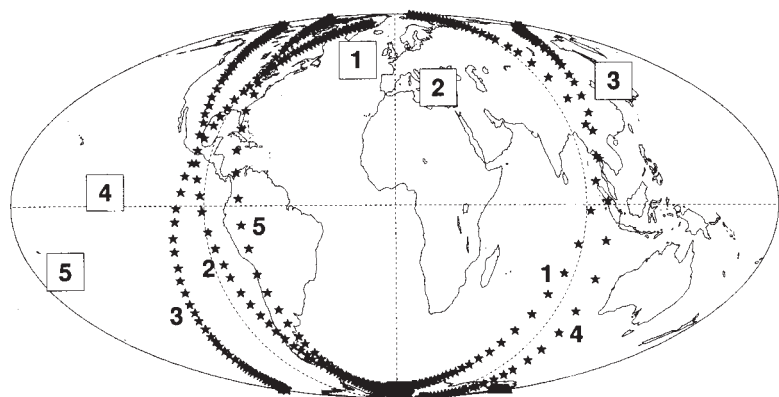


FIG. 4 Plot of synthetic Virtual Geomagnetic Pole (VGP) paths for the field models in Fig. 3 for five arbitrarily chosen sites. The figures in boxes mark and label the site locations; the smaller stars show the VGPs calculated for 301 equal time-steps in the course of one field reversal, with each path labelled by the appropriate site number. The paths are heavily site-dependent because the field does not reverse simply through an equatorial dipole: by the symmetry of the field, sites equatorially anti-symmetric or rotated 180° in longitude have antipodal paths: thus sites on the equator undergo an instantaneous reversal of the VGP. The approximate symmetry observed about the meridian plane $\phi=0$ gives a similar effect: when the site is close to 0° or 180° , small changes in its longitude yield dramatic shifts of the transition path. Thus, an approximate quadrant-type behaviour is observed: sites approximately northeast or southwest of the point (0° N, 0° E) produce normal-to-reverse transition paths lying in one hemisphere, whereas sites in the remaining two quadrants produce an antipodal path.

might be expected to resemble a mix of the two field morphologies found here, and the reversal transition field would resemble the oscillatory solution.

Our mechanism differs from that of Parker and Levy^{8,10}, in which reversals are controlled by fluctuations in the latitude of the α effect rather than by strength of meridian circulation. So far no 3D solutions have been found for their case and no transition fields are available.

There are very few examples of geodynamo models that are sufficiently well developed to allow comparison with observation. We have found two simple physical effects that determine

the field morphology and its time dependence: that downwelling causes concentration of flux and dynamo waves cause flux to migrate poleward. These effects are quite general and are expected to arise in a large range of dynamo models, both kinematic and dynamic. In combination they produce a striking effect: the VGP paths lie close to the longitudes of the flux concentrations of the neighbouring stationary fields. Some recent palaeomagnetic results^{6,7} show this happening on the Earth, where the present-day flux concentrations reside near longitudes 90° W and 90° E, the same longitudes as outlined by many VGP reversal transition paths. □

Received 15 September 1993; accepted 17 January 1994.

- Hutchinson, K. A. & Gubbins, D. *Geophys. J. Int.* **116**, 304–320 (1994).
- Gubbins, D. in *Flow and Creep in the Solar System: Observations, Modelling and Theory* (eds Stone, D. B. & Runcorn, S. K.) 97–111 (Kluwer, Dordrecht, 1993).
- Gubbins, D. *Nature* **326**, 167–169 (1987).
- Merill, R. T. & McElhinny, M. W. in *The Earth's Magnetic Field: Its History, Origin and Planetary Perspective* Ch. 5 (Academic, San Diego, 1983).
- Gubbins, D. & Kelly, P. *Nature* **365**, 829–832 (1993).
- Tric, E. C. et al. *Phys. Earth planet. Inter.* **65**, 319–336 (1991).
- Clement, B. M. *Earth planet. Sci. Lett.* **104**, 48–58 (1991).
- Levy, E. H. *Astrophys. J.* **171**, 621–633 (1972).
- Levy, E. H. *Astrophys. J.* **171**, 635–642 (1972).
- Parker, E. N. in *Cosmical Magnetic Fields: Their Origin and Their Activity* Ch. 19 (Clarendon, Oxford, 1979).
- Bullard, E. C. & Gellman, H. *Proc. R. Soc. A* **247**, 213–278 (1954).
- Lilley, F. E. M. *Proc. R. Soc. A* **316**, 153–167 (1970).
- Gibson, R. D. & Roberts, P. H. in *The Application of Modern Physics to the Earth and*

- Planetary Interiors* (ed. Runcorn, S. K.) 577–602 (Wiley Interscience, New York, 1969).
- Gubbins, D. *Phil. Trans. R. Soc. A* **274**, 493–521 (1973).
- Dudley, M. L. & James, R. W. *Proc. R. Soc. A* **425**, 407–429 (1989).
- Braginsky, S. I. *Soviet Phys. JETP* **20**, 726–735 (1965).
- Roberts, P. H. *Phil. Trans. R. Soc. A* **271**, 663–697 (1972).
- Moffatt, H. K. in *Magnetic Field Generation in Electrically Conducting Fluids* Ch. 9 (Cambridge Univ. Press, 1978).
- Malkus, W. V. R. & Proctor, M. R. E. *J. Fluid Mech.* **67**, 417–443 (1975).
- Jennings, R. L. & Weiss, N. O. *Mon. Not. R. astr. Soc.* **252**, 249–260 (1991).
- Hagee, V. L. & Olson, P. *J. geophys. Res.* **96**, 11673–11687 (1991).
- Hollerbach, R. & Jones, C. A. *Nature* **365**, 541–543 (1993).
- Kumar, S. & Roberts, P. H. *Proc. R. Soc. A* **344**, 235–238 (1975).
- Tric, E. C. et al. *J. geophys. Res.* **97**, 9337–9351 (1992).
- Laj, C., Mazaud, A., Weeks, R., Fuller, M. & Herrero-Bervera, E. *Nature* **351**, 447 (1991).
- Gubbins, D. & Coe, R. *Nature* **362**, 51–53 (1993).
- Honkura, Y., Iijima, T. & Marsushima, M. *J. Geomag. Geoelectr.* **44**, 931–941 (1992).
- Gubbins, D. & Bloxham, J. *Nature* **325**, 509–511 (1987).

Antiquity of *Homo sapiens* in China

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TEN years ago a well-preserved skull of an early form of *Homo sapiens* was unearthed from Pleistocene cave deposits at the Jinniushan site in China. Here we present electron-spin resonance (ESR) and uranium-series dates from five fossil animal teeth collected from the hominid locality. The minimum ESR ages (195–165 kyr) are about 50 kyr younger than the uranium-series dates. Taken together, the results suggest an age of about 200 kyr or older for the Jinniushan skull, making it among the oldest *H. sapiens* material found in China, and almost as old as some of the latest Chinese *H. erectus*. This raises the possibility of the coexistence of the two species in China. The morphology of the skull suggests a strong local component of evolution, consonant with the 'multi-regional continuity' model of the evolution of *H. sapiens*.

The Jinniushan site, an ancient cave locality, is located in Yingkou County, Liaoning Province (40° 15' N, 122° 30' E). The fossiliferous deposit is about 12 m thick. Near the bottom of the deposits a human skull (Fig. 1), together with the vertebrae, ribs, pelvis, patella and limb bones of a single individual were unearthed in 1984¹. The skull's cranial capacity was about 1,390 ml and the thickness of the vault was 4.5 mm on average; it was considered to be close to the skull of early or archaic *Homo sapiens*, for example the Dali skull². The cranial capacity of the anew reconstructed skull has since been remeasured as 1,260 ml (Z. E. Lü, personal communication). The general features of the skull, such as a broad nose bridge, shovel-shaped incisors and prominent cheekbones, differentiate it from the European and African hominid and fit a model of local evolution.

Our early work on U-series dating assigned an age range of 230–300 kyr³ to the layer VI where the skull was found. Although this date correlates well with the Jinniushan's fauna, which includes *Macaca robustus*, *Trogontherium sp.*, *Megalo-*

ceros pachyosteus, *Dicerorhinus mercki* and *Microtus brandtioides*, and suggests an antiquity of Middle Pleistocene, some anthropologists doubt whether *Homo sapiens* could have existed so early. As correct dating is necessary for the understanding of human evolution in China, it is important to crosscheck U-series ages with those from other dating techniques.

Potassium-argon or fission track methods are inapplicable because of the lack of any contemporary volcanic materials. In the age range concerned, ESR dating of fossil enamel may be the only alternative to U-series determination in terms of dating accuracy; however, these two methods are not completely independent as both are based on the assumption of uranium uptake. Here we have dated five additional Jinniushan animal tooth samples using dentine for U-series dating and enamel as for ESR dating.

ESR dating treats the sample as a dosimeter that records the accumulated radiation dose De . The age of the sample, t , is expressed as

$$De = \int_0^t D(t) dt$$

where $D(t)$ is the annual dose rate. As in practice there are dozens of factors that may affect correct age determination (see



FIG. 1 Jinniushan skull: *Norma lateralis*.

TABLE 1 U-series and ESR dating results from five fossil teeth from Jinniushan, Liaoning, China

Sample number	U-series ages of dentine		ESR ages of enamel						
	^{230}Th ages (kyr)	$^{231}\text{Pa}/^{235}\text{U}$	D_e (Gy)	U (p.p.m.) enamel	U (p.p.m.) dentine	$^{234}\text{U}/^{238}\text{U}$	d (mm)	EU ages (kyr)	LU ages (kyr)
91001	237 ± 16	1.10 ± 0.06	708 ± 71	1.17 ± 0.29	60.1 ± 2.0	1.47	2.3	192 ± 23	284 ± 30
91002	197 ± 36	0.998 ± 0.04	572 ± 57	1.47 ± 0.37	71.3 ± 2.1	1.24	2.7	165 ± 20	248 ± 26
91003	198 ± 14	1.07 ± 0.06	675 ± 68	1.13 ± 0.28	71.0 ± 2.1	1.22	2.7	195 ± 23	294 ± 31
84070	304 ± 54 –36	1.05 ± 0.06	998 ± 100	3.00 ± 0.50	59.8 ± 1.7	1.35	2.2	193 ± 23	302 ± 32
84071	258 ± 32 –26	0.989 ± 0.05 6	799 ± 80	2.23 ± 0.40	58.7 ± 1.5	1.35	2.2	188 ± 22	276 ± 29
Mean	237							187	281

For D_e determination, enamel layers were first scraped and ground. For each sample, eight equal aliquots of 200 mg (size 100–150 μm) were γ -irradiated up to 5 kGy, as calibrated with alanine dosimeters. ESR spectral measurements were made on a Bruker ER-200D-SRC X-band ESR spectrometer under the following conditions: ambient temperature; microwave power, 2 mW; field modulation, 2 Gpp; modulation frequency, 100 kHz. Peak height at $g=2.0018$ was taken as the ESR intensity, and the standard deviation for repeated sample filling and measuring is ~2–3%. For annual dose rate $D(t)$ determination, α -particle spectrometry and laser fluorescence methods were used to measure the U, Th and K contents and the uranium isotope ratios of the samples and the surrounding sediment. The U, Th and K contents of the sediments are 2.7 p.p.m., 8.0 p.p.m., and 0.35% respectively. The radon loss was estimated as $L=0.30 \pm 0.15$, based on the empirical determination for three dentine lumps with a Chinese FD-125 radon emanation instrument. The moisture of the samples was estimated as 0.10 ± 0.05 ; 'd' is the average thickness of enamel layers. Nambi and Aitken's Transform Table¹¹ was used to calculate dose rates from the U-series disequilibrium in teeth and β -particle attenuation in enamel layers. The cosmic ray dose contribution was small and estimated to be 0.13 Gy kyr^{-1} .

ref. 4), we will briefly discuss the D_e extrapolation, the α -particle efficiency and the uranium-uptake model.

D_e values were determined by standard saturation-exponential fitting. We observed that as the radiation dose was increased, D_e remained constant at first, but started to increase when irradiated with doses above 7 kGy. Here we use D_e values in the stable range.

When calculating $D(t)$ values, the commonly accepted $K\alpha$ value for enamel is 0.15 (refs 5, 6); we determined this value empirically. Very finely powdered enamel samples were obtained by time-controlled settling on aluminium discs. Samples were irradiated with equal doses on the calibrated 721/B α and 733 β irradiators at the TL lab of the Shanghai Museum; one sample was γ -irradiated. A 50 mg specimen of each treated sample was collected. The ESR intensities of these specimens were compared. Because the 3.7 MeV α -particle irradiator was calibrated in terms of track length, we actually measured the relative efficiency of the 3.7 MeV α -particles $K_{3.7}$, or the α value. Assuming that the stopping power of enamel is equal to that of quartz and $K\alpha=0.8K_{3.7}$ (ref. 7), three independent measurements gave an average $K\alpha$ value of 0.227 ± 0.013 , which is higher than 0.15. We used this measured value of 0.227 for $D(t)$ calculation, consequently the ages derived are 10–25 kyr younger than the ages calculated using $K\alpha=0.15$.

The model of early uranium uptake (EU) was used for the $D(t)$ calculation, which presumes that uranium was taken up during a brief period after burial and remained constant through time as in a closed system. An alternative to EU is the linear uptake (LU) model, which assumes an even and continuous uptake of uranium. The ESR age based on the EU model is always younger than the LU age.

The uranium-series dating of fossil samples is based on the EU assumption, and this assumption is tested by comparing the $^{230}\text{Th}/^{234}\text{U}$ and $^{231}\text{Pa}/^{235}\text{U}$ activity ratios of each sample. We have confirmed that this assumption is valid for samples 91002, 84070 and 84072, and is basically applicable to samples 91001 and 91003 (within 2σ error) (Table 1). We therefore applied the EU model to our ESR age calculations, with LU ages listed in Table 1 for comparison. As secondary uranium addition or leakage often occurs in fossil teeth, it is desirable to check the U-uptake assumption in ESR dating.

A computer program has been developed for ESR age calculation. As parameters in ESR dating may be subject to vague

errors, we, following Grün's suggestion⁸, calculated the individual errors caused by each parameter, then synthesized them with the square root to yield the age uncertainty. The final dating results and error assessment are summarized in Table 1.

We draw the following conclusions. (1) The EU ESR ages shown in Table 1 should be taken as minimum ages. They are younger than but not very different from their uranium counterparts. The fact that ESR dating works for fossil teeth is important for dating earlier sites that are beyond the U-series dating limit, for example the Yunxian site where two hominid skulls were discovered in 1990⁹. (2) Because of the high uranium content in these samples, the mean EU and LU ages (187 and 281 kyr respectively) are well apart. We consider that in this case the LU ages are too old to conform to the geological and archaeological evidence. It would be meaningless to compare the LU ESR ages with U-series ages, because they are based on mutually exclusive assumptions. (3) The new dating results are close to the lower limit of the early published dates for the Jinniushan Site (230–300 kyr), suggesting that the Jinniushan skull dates to about 200 kyr or possibly earlier. Together with Dali and Chaohu hominid fossils, they are the earliest fossils of *Homo sapiens* found in China and are almost as old as some of the latest *Homo erectus*, such as the Skull V in the upper stratum of the Zhoukoudian Site and the Hexian skull. The dating results of these palaeoanthropological sites suggest a possible coexistence of two species of *Homo* in China¹⁰ and also make comparative study of Asian and non-Asian hominids of the same age possible. □

Received 29 March; accepted 1 December 1993.

1. Lü, Z. E. *Liaohai Wenwu Xuekai* No 1 44–55 (1989) (in Chinese).
2. Wu, R. K. *Acta anthropologica sinica* **7**, 97–101 (1988) (in Chinese).
3. Chen, T. M. & Yuan, S. X. *Archaeometry* **30**, 59–76 (1988).
4. Grün, R. *Appl. Radiat. Isot.* **40**, 1045–1055 (1989).
5. Grün, R. *Sonderveröffentlichungen des Geologischen Instituts der Universität Köln* **59**, 1–157b (1985).
6. DeCanniere, P., Debuyst, R., Dejeht, F., Apers, D. & Grün, R. *Nucl. Tracks* **11**, 211–220 (1986).
7. Zimmerman, D. E. *Archaeometry* **13**, 29–52 (1971).
8. Grün, R. *Nucl. Track Radiat. Meas.* **18**, 143–153 (1991).
9. Li, T. Y. & Etlér, D. A. *Nature* **357**, 404–407 (1992).
10. Chen, T. M. & Zhang, Y. Y. *World Archaeol.* **23**, 147–154 (1991).
11. Nambi, K. S. V. & Aitken, M. J. *Archaeometry* **28**, 202–205 (1986).

ACKNOWLEDGEMENTS. This project is supported by the NSF of China. We thank W.D. Wang of the Shanghai Museum for his help in determining the $K\alpha$ value, and Z. E. Lü for Fig. 1.

Neurogenesis in an adult insect brain and its hormonal control

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HORMONALLY mediated changes in behaviour patterns are found in many animal species, including adult insects¹⁻⁴, raising the possibility that the neurological modifications linked with such changes could be mediated by morphogenetic hormones. Neuronal plasticity and neurogenesis in the adult brain have rarely been reported in vertebrates⁵⁻¹⁰, and in adult insects only modifications of mushroom body volumes or fibres have been described¹¹⁻¹⁴. Here we report the presence of undifferentiated cells, located dorsally in mushroom bodies of the house cricket *Acheta domesticus*, that persist, divide and give rise to cortical interneurons during adult life. Furthermore, juvenile hormone, which induces oviposition behaviour in adult crickets^{2,3}, acts on neurogenesis in this cell cluster. Females deprived of juvenile hormone showed a significant decrease in cell division, which was reversed by hormone injection. Production of juvenile hormone therefore appears to be a major factor in the adaptation of the brain to concomitant physiological and behavioural changes in adult insects.

To determine whether neuroanatomical changes occur during adult life, we focused our study on the intrinsic neurons of the mushroom bodies of *A. domesticus*, as this paired structure represents the main integrative centre of the insect brain¹⁵⁻¹⁸. Each mushroom body consists of a neuropile and a cortex of intrinsic neurons (the globuli cells or Kenyon cells), densely packed in the calyces. A cytological examination revealed a cluster of two types of undifferentiated cells in the dorsal region of mushroom

body cortices (Fig. 1a). Although no cell division occurred in the Kenyon cell mass, mitoses were observed in both types of undifferentiated cells (Fig. 1b, c). According to the pattern in immature stages^{19,22}, the larger cells can be referred to as neuroblasts that produce, by asymmetric division (Fig. 1c), smaller ganglion mother cells. These subsequently divide symmetrically to neurons (Fig. 1b), which differentiate as Kenyon cells¹⁹⁻²². As neuroblasts are said to disappear before the adult moult²⁰⁻²⁴, we used 5-bromodeoxyuridine (BrdU) as a marker of DNA synthesis²⁵ to verify our initial observations. BrdU was first incorporated *in vitro* into cephalic ganglia freshly dissected from 1-3-day-old females. Incubation for 5 h in a tissue culture medium containing BrdU resulted in dense labelling of the undifferentiated cells (Fig. 2a). To determine the fate of cells arising from the mitotic divisions, another set of experiments was designed using *in vivo* BrdU incorporation. Females received three injections of BrdU at 24 h intervals following emergence, and cytological analysis of brains was performed on days 3, 5 and 7. As the time between injection and dissection increased, the label of the dividing cells from the proliferative area progressively disappeared. It was hardly visible 5 days after the last BrdU injection, indicating that DNA replication had continued. Furthermore, the labelled cells appeared progressively farther and farther away from the undifferentiated area, and had the size and shape that typifies neuronal cell bodies that are ascribed to intrinsic neurons of mushroom bodies (Fig. 2b). From this key observation we concluded that neurogenesis occurs in adult *A. domesticus*, where persisting neuroblasts serve as progenitors of new neural cells.

The incidence of neurogenesis was estimated from emergence to day 11 by counting the total number of neuroblasts and ganglion mother cells in M phase. The putative role of juvenile hormone on neurogenesis was checked by comparing the number of cell divisions in control females and in similar aged individuals that had been allatectomized before the adult moult, and thus lacked juvenile hormone. Females, ovariectomized before the adult moult, served as additional controls as they had juvenile hormone titres similar to those of intact adults²⁶. It is evident that although the mean incidence of cell division for all ages examined did not differ ($P=0.323$) between controls (20.9 ± 1.7) and ovariectomized females (20.3 ± 1.9), it was markedly reduced in allatectomized individuals (12.4 ± 0.6 , $P=0.001$) (Fig. 3). The difference was most pronounced between days 2 and 5 (Fig. 3), when juvenile hormone titre increases in control females²⁷. These results strongly implicate a hormonal regulation of neurogenesis, so we injected juvenile hormone into newly emerged and 10-day-old allatectomized females. These individuals were killed 3 days after treatment and, as expected, there was a significant increase in the number of cell divisions for both 3-day-old ($P=0.003$) and 13-day-old females ($P=0.05$)

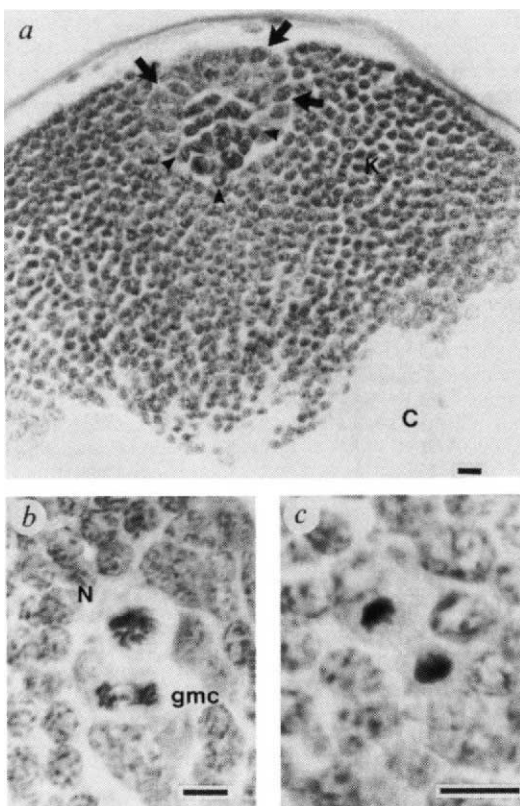
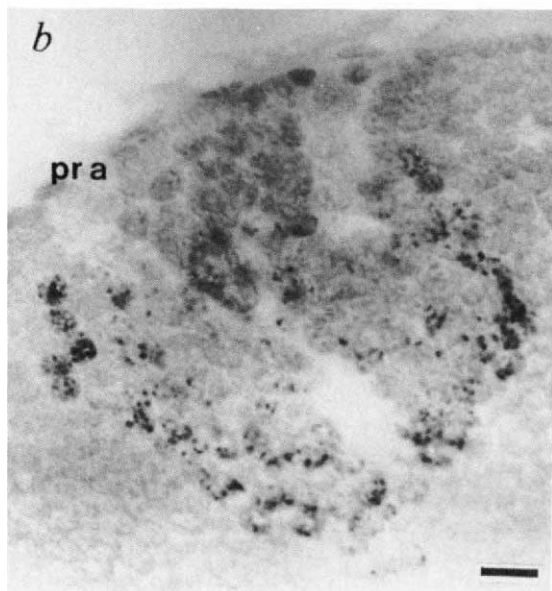
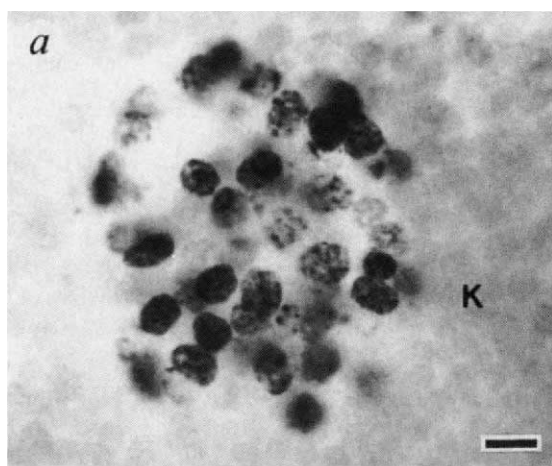


FIG. 1 Frontal sections through a mushroom body of *A. domesticus*. **a**, Cluster of quiescent undifferentiated cells at the apical part of the cortex in a 3-day-old adult female. Neuroblasts (arrows) are large light-staining cells with ovoid nuclei (12 µm long, 7.5 µm wide); they surround ganglion mother cells (arrowheads), which are smaller round cells with denser karyoplasm and cytoplasm (nuclei; diameter 7 µm). Typical Kenyon cells (K) (nuclear diameter, 6 µm) are densely packed in the calyx (C). Scale bar, 10 µm. **b**, Proliferating neuroblast and ganglion mother cell in a 2-day-old adult female. Based on their size and orientation, the metaphase and anaphase cells were identified as neuroblast (N) and ganglion mother cell (gmc), respectively. Scale bar, 10 µm. **c**, Asymmetrical neuroblast mitosis. The smaller cell will become a ganglion mother cell. Scale bar, 10 µm.

METHODS. Cerebral ganglia were quickly dissected in saline, then fixed for 6 h in Carnoy's fixative. After 3×24 h washing in 95% ethanol and 3×24 h in 1-butanol, tissues were embedded in paraffin and cut in 6-µm serial sections. Sections were deparaffined, rehydrated and treated according to the method of Feulgen-Rossenbeck³³. DNA was hydrolysed using 6 N HCl for 60 min at room temperature. Sections were counterstained in 0.4% Picroindigo carmin in a saturated solution of picric acid, dehydrated, cleared and mounted in DepeX (Merck). Micrographs were taken using a Zeiss photomicroscope.



◀ FIG. 2 Identification of dividing cells in a mushroom body of *A. domesticus* with 5-bromodeoxyuridine. *a*, Disc-shaped pattern of BrdU-labelled nuclei at the apex of mushroom body cortex, surrounded by unlabelled interneurons (K, Kenyon cells). Dorsal view of a whole-mount preparation of a 2-day-old adult female after 5 h *in vitro* labelling. Scale bar, 10 μ m. *b*, Frontal section through a mushroom body after *in vivo* labelling. In this 5-day-old adult female sacrificed 3 days after the third BrdU daily injection, the large cells of the proliferative area (pra) presented only a few label traces. By contrast, smaller labelled nuclei (arrowheads), undistinguishable from authentic interneurons, invaded the cortical part of the mushroom body. Scale bar, 10 μ m. Observation of other parts of the brain and of suboesophageal ganglion failed to reveal any other labelled neuron. Some small, usually elongated, nuclei located in the perineurial sheath, or scattered at the periphery of the neuropile were undoubtedly perineurial and glial cells.

METHODS. DNA replication was detected using BrdU²⁵. For *in vitro* incorporation, freshly dissected cephalic ganglia were incubated for 1.5–5 h at 30 °C in tissue culture medium 199 with Hank's salts and L-glutamine (Gibco), containing 20 mg ml⁻¹ Ficol and 1.5 mg ml⁻¹ BrdU (Sigma). For *in vivo* studies, 10 μ l BrdU dissolved in saline (15 mg ml⁻¹) was injected at 1 h, 24 h and 48 h post-emergence, and animals were examined respectively at 3, 5 and 7 days. The cephalic ganglia were fixed for 2 h with Carnoy's fixative and treated either as whole mounts or paraffin-sectioned preparations according to ref. 21.

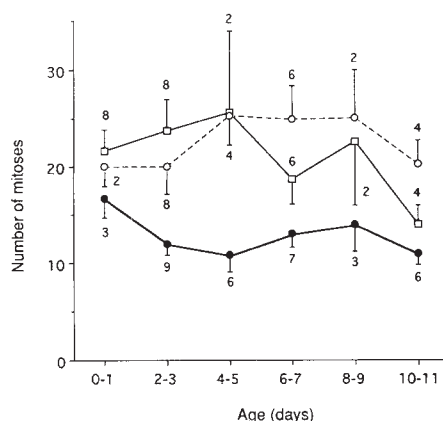
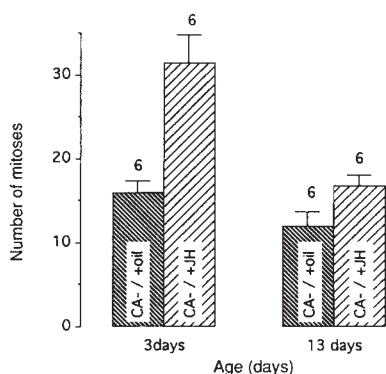


FIG. 3 Effects of juvenile hormone deprivations on the mitotic activity observed in mushroom bodies of *A. domesticus*. Whereas the mean number of mitoses occurring during the 11 first days of adult life did not significantly differ between controls (square symbols; 20.9 ± 1.7) and ovariectomized females (open circles; 20.3 ± 1.9), the mean number of cell divisions was markedly reduced (12.4 ± 0.6 , $P = 0.001$; filled circles) after removal of the source of juvenile hormone, the corpora allata (allatectomy). The largest differences between control and allatectomized females were observed between days 2 to 5.

METHODS. Analysis of mitoses was performed blind on serial 6 μ m sections of brains from 0- to 11-day-old adult females (see Fig. 2 for cytological methods). Allatectomies and ovariectomies took place during the last larval instar. The data are expressed as mean values $\pm$ s.e.m. and the numbers indicate sample numbers. Means were compared using the Mann-Whitney *U* test ($P < 0.05$).



◀ FIG. 4 Stimulatory action of juvenile hormones (JH) on mushroom body mitotic activity of *A. domesticus*. Significant increases in cell division rates were observed after JH injection; they were more pronounced in 3-day-old ($P = 0.003$) than in 13-day-old ($P = 0.05$) females.

METHODS. Newly emerged and 10-day-old allatectomized females were injected with 10 μ l paraffin oil (CA- / +oil), or with 100 μ g JH III emulsified in 10 μ l paraffin oil (CA- / +JH). Brains were dissected 3 days later and treated for study of serial section (Fig. 3) as described in the legend of Fig. 2. Mitotic activity was analysed as described and data expressed as mean values $\pm$ s.e.m. Numbers indicate the number of samples, and significance of differences was determined using the Mann-Whitney *U* test ($P < 0.05$).

compared to allatectomized individuals of the same age (Fig. 4). We concluded that juvenile hormone could affect adult insect brain structure through neurogenesis.

In adult song birds, steroids elicit major structural and functional changes²⁸ but not neurogenesis²⁹. However, although plasticity has been described in adult insects^{30,31}, regulation of neuronal remodelling through endocrine signals was presumed to occur only during pre-adult stages of development^{21,32}. Our findings indicate that plasticity also occurs in the brain of adult insects, as the appearance of new neurons implies the formation of new synapses. The persistent neurogenesis in *Acheta* mush-

room bodies convincingly demonstrates that substantial reorganization may occur in response to changing levels of circulating juvenile hormone during adult life. These hormone-mediated changes could provide the neuroanatomical basis for hormone-controlled expression of oviposition behaviour in the house cricket.

Our findings raise a number of questions concerning the importance of adult neurogenesis. Is it a widespread phenomenon in invertebrates or is it restricted to species with long-lived adults that have complex reproductive cycles? Furthermore, as our preliminary observations indicate that mitosis also occurs in

A. domesticus males, interspecific and intersex comparisons will provide a better understanding of the interrelationships between hormones, neural growth and the ontogeny of behaviour in adult invertebrates. □

Received 9 September 1993; accepted 5 January 1994.

- Loher, W. Z. *vergl. Physiol.* **53**, 277–316 (1966).
- Renucci, M., Cherkaoui, L., Rage, P. & Strambi, A. C. *Acad. Sci.* **307**, 729–733 (1988).
- Renucci, M., Cherkaoui, L. & Strambi, A. in *Insect Juvenile Hormone Research: Fundamental and Applied Approaches* (eds Mauchamp, B., Couillaud, F. & Baehr, J. C.) 147–163 (INRA, Paris, 1992).
- Robinson, G. E., Page R. E. Jr, Strambi, C. & Strambi A. *Science* **246**, 109–112 (1989).
- Altman, J. *Science* **135**, 1127–1128 (1962).
- Polenov, A. L., Chetverukhin, V. K. & Jakovleva, I. V. Z. *Mikrosk anat. Forsch.* **85**, 513–532 (1972).
- Kaplan, M. S. & Hinds, J. W. *Science* **197**, 1092–1094 (1977).
- Birse, S. C., Leonard, R. B. & Coggeshall, R. E. J. *comp. Neurol.* **194**, 291–301 (1980).
- Bayer, S. A., Yackel, J. W. & Puri, P. S. *Science* **216**, 890–892 (1982).
- Goldman, S. A. & Nottebohm, F. *Proc. natn. Acad. Sci. U.S.A.* **80**, 2390–2394 (1983).
- Bieber, M. & Fuldner, D. *Naturwissenschaften* **66**, 426 (1979).
- Coss, R. G., Brandon, J. G. & Globus, A. *Brain Res.* **192**, 49–59 (1980).
- Technau, G. M. J. *Neurogenet.* **1**, 113–126 (1984).
- Withers, G. S., Fahrbach, S. E. & Robinson, G. E. *Nature* **364**, 238–240 (1993).
- Huber, F. Z. *vergl. Physiol.* **44**, 60–132 (1984).
- Erber, J., Masuhr, T. & Menzel, R. *Physiol. Ent.* **5**, 343–358 (1980).
- Heisenberg, M., Borst, A., Wagner, S. & Byers, D. J. *Neurogenet.* **2**, 1–30 (1985).
- Han, P. L., Levin, L. R., Reed, R. R. & Davis, R. *Neuron* **9**, 619–627 (1992).
- Panov, A. A. *Rev. Entomol. URSS* **45**, 326–340 (1966).
- Nordlander, R. H. & Edwards, J. S. *Wilhelm Roux Arch. dev. Biol.* **164**, 247–260 (1992).
- Truman, J. W. & Bate, M. *Dev. Biol.* **125**, 145–157 (1988).
- Ito, K. & Hotta, Y. *Dev. Biol.* **149**, 134–148 (1992).
- Edwards, J. S. *Adv. Insect Physiol.* **6**, 97–137 (1970).
- White, K. & Kankel, D. R. *Dev. Biol.* **65**, 296–321 (1978).
- Gratzner, H. G. *Science* **218**, 474–475 (1982).
- Renucci, M., Strambi, C., Strambi, A., Augier, R. & Charpin P. *Gen. comp. Endocrinol.* **78**, 137–149 (1990).
- Renucci, M. & Strambi, C. *Experientia* **39**, 618–620 (1983).
- de Voogd, T. J. & Nottebohm, F. *Science* **214**, 202–204 (1981).
- Brown, S. D., Johnson, F. & Bottjer, S. W. J. *Neurosci.* **13**, 2024–2032 (1993).
- Hoy, R. R., Nolen, G. & Casaday, G. B. *Proc. natn. Acad. Sci. U.S.A.* **82**, 7772–7776 (1985).
- Huber, F. J. *comp. Physiol. A* **161**, 583–604 (1987).
- Truman, J. W. J. *Neurobiol.* **23**, 1404–1422 (1992).
- Feulgen, R. & Rossenbeck, H. Z. *physiol. Chem.* **135**, 203 (1924).

ACKNOWLEDGEMENTS. We thank A. Yvinnec, P. Weber, P. Charpin and R. Augier for technical assistance, J. W. Truman for discussions and B. Stay, A. Woodhead and J. McNeil for critical comments and for help in preparing the manuscript.

Glial contributions to excitatory neurotransmission in cultured hippocampal cells

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ALTHOUGH many glial cells possess neurotransmitter receptors and transporters^{1–5}, little is known about glial participation in neurotransmission. To explore this issue, we recorded neuronal autaptic^{6,7} and glial responses from cultured hippocampal single-neuron micro-islands^{6,7}. Excitatory synaptic events activate rapid electrogenic glial glutamate transporter currents similar to those elicited by exogenous glutamate in other preparations. We show here that glial transporter responses may be used to sense changes in presynaptic efficacy and that glial uptake helps to remove synaptically released glutamate, thereby contributing to the termination of excitatory synaptic currents under certain conditions. These observations provide a framework for understanding the role of glia in both normal and pathological processes.

Inward glial currents were detected on 88% (91/103) of visually identified single-neuron micro-islands (Fig. 1a, b), displaying a neuronal excitatory autaptic response. In neurons, elicited action potentials were followed by autaptic afterdepolarizations (Fig. 1c and refs 6, 7). Like neuronal afterdepolarizations, voltage-clamped glial cell currents appeared with a short latency following neuronal action potentials (Fig. 1c). Excitatory autaptic currents (e.a.cs) underlying autaptic depolarizations in neu-

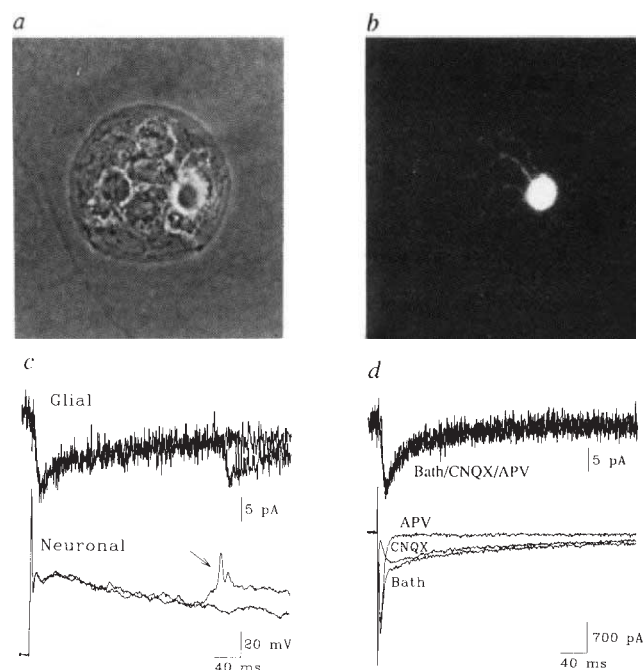
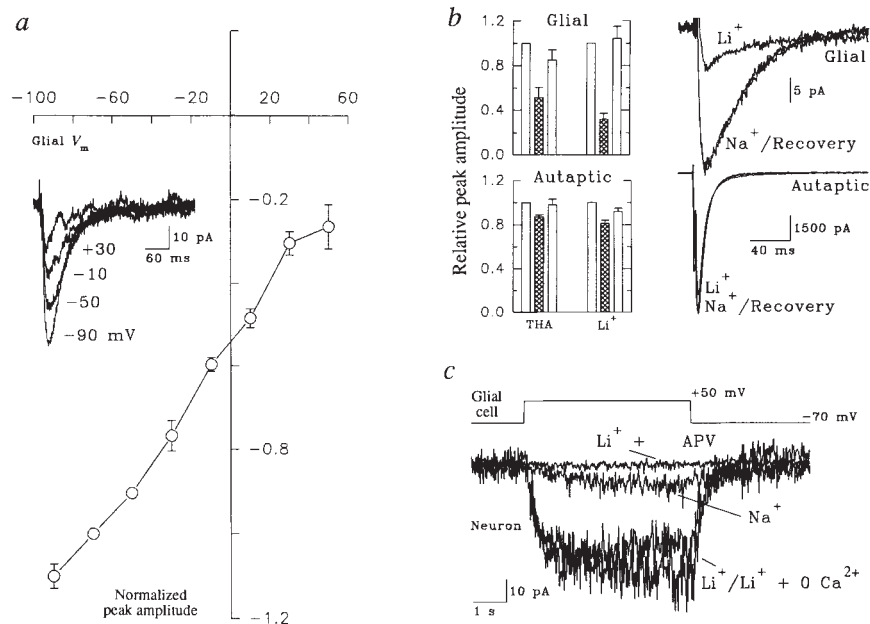


FIG. 1 Micro-island glial cells respond to autaptic transmission. a, Photomicrograph showing a micro-island typical of those used for experiments. Note the single phase-bright neuron and the underlying multinucleated glial micro-island. b, After 3 min of whole-cell recording from the neuron in a with a pipette solution containing 1% Lucifer yellow, neurites were visible extending into the glial substrate. Scale bars for a and b, 20 μ m. c, Two superimposed evoked neuronal action potentials (bottom traces) caused prolonged neuronal autaptic afterdepolarizations^{6,7} and inward currents in an underlying glial cell clamped at -70 mV (top traces). One autaptic afterdepolarization caused a spontaneous action potential (arrow) associated with secondary autaptic and glial responses. d, From the cells shown in c, autaptic responses were elicited using a voltage-clamp protocol⁷. The neuronal membrane potential was stepped from -70 mV to $+20$ mV for 1.5 ms. For this figure, transients associated with voltage steps are intact. For all other figures, transients have been blanked. CNQX (10 μ M) eliminated fast ($\tau_{\text{decay}} = 7.0 \pm 0.7$ ms, $n = 20$) non-NMDA e.a.cs. APV (50 μ M) eliminated slower ($\tau_{\text{decay}} = 74.2 \pm 4.3$ (69%), $\tau_{\text{decay}} = 421 \pm 19$ ms, $n = 20$) NMDA e.a.cs. Neither antagonist affected glial currents. Glial currents exhibited a single exponential decay of 43.1 ± 2.3 ms ($n = 20$). For these experiments bath solutions contained 10 μ M glycine and no added Mg^{2+} .

METHODS. Hippocampal cells were collected from 1–3-day postnatal albino rats¹⁴, plated onto culture dishes coated with 0.15% agarose and sprayed with collagen using a microatomizer^{6,7}. Unless otherwise specified, the external solution contained (mM): 137.5 NaCl, 4 KCl, 3 CaCl_2 , 1 MgCl_2 , 10 glucose, 0.03 APV, 10 HEPES, pH 7.25. Solution exchanges were made with a multibarrel local perfusion system which gave complete solution exchanges in 200–500 ms. Excitatory autaptic currents were recorded using a perforated patch technique²⁷ to inhibit rundown. Pipette solutions contained (mM): 115 potassium gluconate, 10 potassium glutamate, 10 KCl, 5 NaCl, 3 MgCl_2 , 10 HEPES, pH 7.25. Nystatin, Pluronic F127 and dimethyl sulphoxide were present at 300 $\mu\text{g ml}^{-1}$, 250 $\mu\text{g ml}^{-1}$ and 0.35%, respectively. Voltage-clamp recordings of e.a.cs were made using a discontinuous single-electrode voltage-clamp (8–10 kHz switching frequency) to minimize series resistance errors. Glial pipette solutions normally contained (mM): 125 potassium gluconate, 5 NaCl, 0.5 CaCl_2 , 5 EGTA, 10 HEPES, 2 Mg-ATP , pH 7.25. Glial recordings were usually made with an Axopatch 1-D amplifier. Series resistance was compensated 70–90% when the glial membrane potential was displaced from -70 mV. Micro-islands typically ranged over 50–200 μ m in diameter and were used for experiments 8–15 days after plating. Stepping the membrane voltage in one glial cell produced a similar step in the membrane potential of a current-clamped partner glial cell on the opposite side of the micro-island with a steady-state error of 5–10% ($n = 4$). This indicates that a reasonable voltage-clamp of an entire glial micro-island could be achieved and is consistent with extensive dye coupling of type I astrocytes²⁸. Unless otherwise indicated, all neuronal and glial traces represent averages of 2–15 responses. Values in text and figures represent mean $\pm$ standard error.

FIG. 2 Glial responses exhibit characteristics of a Glu transporter. **a**, The graph shows the current/voltage relationship for glial responses. Symbols represent peak currents from four cells normalized to responses at -70 mV. The inset shows responses from one glial cell at the indicated membrane potentials. Glial responses were evoked as in Fig. 1d. **b**, The histograms (left) show the effects of threo-3-hydroxyaspartate ($100 \mu\text{M}$, $n=7$) and substitution of Li^+ for Na^+ ($n=8$) on peak glial currents (top histograms) and non-NMDA e.a.cs (bottom histograms). The left, middle and right bars in each triplet represent control, experimental and recovery conditions, respectively, normalized to control values. The traces (right) show the effects of Li^+ on glial currents and non-NMDA e.a.cs for one micro-island. Autaptic currents were evoked as in Fig. 1d. Glial holding potential was -70 mV. **c**, Glial depolarization induces neuronal NMDA receptor-mediated currents. The top trace represents the command potential of a voltage-clamped glial cell. The bottom traces show the response of a neuron voltage-clamped at -70 mV on the same micro-island. For these experiments the external solution contained $10 \mu\text{M}$ glycine and no added Mg^{2+} or APV. Also, 10 mM potassium gluconate in the glial pipette solution was replaced with 10 mM potassium glutamate. The neuronal current induced by glial depolarization was enhanced by substitution of Li^+ for Na^+ . Whereas bath solution containing no added divalent cations reduced autaptic responses to undetectable levels in 4/4 neurons, the



neuronal current induced by glial depolarization was largely Ca^{2+} -independent, because in 9 glial/neuronal pairs the average neuronal current induced by glial depolarization in LiCl ($3 \text{ mM } \text{Ca}^{2+}$) was 51 pA , compared with 47 pA in the same bath solution containing no added Ca^{2+} . APV ($50 \mu\text{M}$) eliminated the current in all cells tested ($n=11$).

rons were composed of both fast non-*N*-methyl-D-aspartate (non-NMDA) receptor-mediated components and slow NMDA receptor-mediated components (Fig. 1d). Glial responses were not mediated by either of these receptor types, as glial currents were unaffected by $10 \mu\text{M}$ 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; $9.0 \pm 6\%$ decrease, $n=4$) or by $50 \mu\text{M}$ D-amino-5-phosphonovaleric acid (APV; $2.0 \pm 7\%$ increase, $n=4$), treatments which abolished the two components of e.a.cs (Fig. 1d).

In retinal Müller cells and in cultured type I cerebellar astrocytes, exogenous glutamate (Glu) activates electrogenic uptake currents that have characteristic features^{1,3-5}. Like these uptake currents, autaptically associated micro-island glial currents remained inward even at glial membrane potentials as positive as $+50$ mV (Fig. 2a). Additionally, the glutamate uptake inhibitor threo-3-hydroxyaspartate (THA) induced small (10 – 100 pA) inward glial currents⁷ and reversibly reduced autaptically associated glial currents with a 50% inhibitory concentration (IC_{50}) of $4.6 \mu\text{M}$. However, hydroxyaspartate produced a maximal inhibition of only 48% , indicating that there is a component of glial responses that is insensitive to this compound. Although hydroxyaspartate slightly depressed peak non-NMDA receptor-mediated e.a.cs, this effect was much smaller than the inhibition of glial currents (Fig. 2b). Another uptake inhibitor, (–)-transpyrrolidine-2,4-dicarboxylic acid (PDC)⁸, also depressed glial currents ($61 \pm 8\%$ with $100 \mu\text{M}$; $n=5$). Lithium, while permeant to voltage-gated sodium ion channels⁹ and to ionotropic glutamate receptors¹, inhibits Na^+ -dependent glutamate uptake^{1,3,4}. Replacement of Na^+ with Li^+ in the extracellular solution depressed glial responses ($68 \pm 5\%$, $n=8$), with only a small inhibition of autaptic currents (Fig. 2b).

If autaptically associated glial currents represent activation of electrogenic transporters, then under appropriate conditions⁵ reverse uptake should produce glial Glu efflux. In a majority (13/22) of single-neuron micro-islands, depolarization of glia to $+50$ mV induced a slowly rising neuronal current (Fig. 2c). Replacement of extracellular Na^+ with Li^+ enhanced neuronal responses to glial depolarization by 6.6 ± 2.1 -fold ($n=8$). D-

Amino-5-phosphonovaleric acid ($50 \mu\text{M}$) abolished the neuronal response, indicating that it was mediated by NMDA receptors. Unlike autaptic responses, however, these currents were not dependent on extracellular calcium (Fig. 2c). The ion dependence of the neuronal response and the dependence on glial membrane potential are consistent with these currents being mediated by release of glutamate through glial reverse transport⁵.

These studies suggest that glial responses primarily represent glutamate uptake following autaptic release of transmitter. However, in 5/11 cells a small inward current was activated by neuronal stimulation when cells were switched to a bath solution containing no added Ca^{2+} , $4 \text{ mM } \text{Mg}^{2+}$ and 0.5 mM EGTA, which abolished autaptic release. This current probably represents ion buffering in response to neuronal stimulation¹⁰. Because of its small amplitude ($<10\%$ of peak glial responses in the standard bath solution), this residual current was not studied further.

Traditionally, quantal analysis has been used to assign presynaptic versus postsynaptic loci of synaptic change, though use of quantal analysis at central nervous system synapses has been criticized¹¹. Glia might provide model-independent sensors of presynaptic changes, because treatments that modulate glutamate release should produce similar changes in autaptic and glial uptake responses. Manipulation of extracellular divalent cation concentrations to alter transmitter release¹² revealed a nearly linear relationship between peak autaptic and glial currents over the range of 1.5 – $3.0 \text{ mM } \text{Ca}^{2+}$ (Fig. 3a). Similarly, the GABA_B agonist (–)-baclofen ($50 \mu\text{M}$)¹³ reversibly reduced both non-NMDA e.a.cs ($58 \pm 5\%$) and glial responses ($66 \pm 5\%$, $n=8$).

Like CNQX and APV (Fig. 1d), two other presumed postsynaptic manipulations failed to change glial responses consistently. Increasing the neuronal holding potential from -70 to -100 mV increased the amplitude of non-NMDA e.a.cs ($35 \pm 4\%$ increase) but had no effect on glial currents ($2.0 \pm 4\%$ decrease, $n=9$; Fig. 3b). The non-competitive non-NMDA antagonist GYKI 52466 ($50 \mu\text{M}$)¹⁴ depressed autaptic non-NMDA responses ($83 \pm 3\%$ depression) but produced no consistent change in glial currents

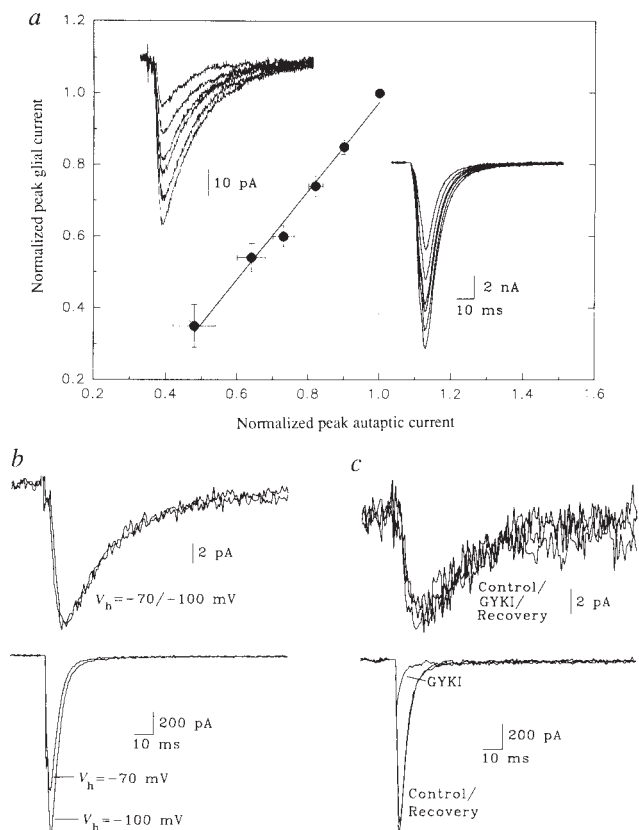


FIG. 3 Presynaptic modulation causes similar changes in autaptic and glial currents; postsynaptic manipulations fail to alter glial responses. **a**, The graph depicts peak glial currents plotted against peak non-NMDA e.a.cs ($n=7$), as Glu release was decreased by lowering $[Ca^{2+}]_o$ and raising $[Mg^{2+}]_o$. The largest currents were obtained with 3 mM Ca^{2+} and 1 mM Mg^{2+} . Subsequent responses resulted from lowering Ca^{2+} and raising Mg^{2+} in 0.3 mM steps. Data were normalized to responses in 3 mM Ca^{2+} and 1 mM Mg^{2+} , and the line represents a linear regression fit to the data ($r=0.99$). The upper and lower insets depict glial and autaptic responses, respectively, from one representative pair. **b**, Changing the neuronal holding potential (V_h) before and after autaptic stimulation from -70 to -100 mV increased the peak non-NMDA e.a.c. but failed to alter the glial current. **c**, The noncompetitive non-NMDA receptor antagonist GYKI 52466 (GYKI; 50 μ M) nearly abolished non-NMDA e.a.cs but had no effect on glial currents. All bath solutions contained 30 μ M APV; autaptic currents were elicited as in Fig. 1d; glial holding potential was -70 mV.

($11 \pm 8\%$ depression, $n=3$; Fig. 3c). The results shown in Fig. 3 suggest that glial currents could be used as corroborative evidence of presynaptic modulation; however, a change in glial currents could indicate modulation of uptake in addition to or rather than a presynaptic change. Also, the small amplitude of glial uptake currents would make detection of small changes in glutamate release or detection of single quanta difficult.

It has been estimated that glutamate is present at high concentrations in the synaptic cleft for less than 2 ms following synaptic release¹⁵. It is unclear whether uptake plays a significant role in clearance of glutamate, as theoretical predictions suggest that diffusion alone may be capable of rapid clearance¹⁶, and previous studies suggest no effect of uptake inhibitors on decays of excitatory postsynaptic currents (e.p.s.cs)^{17,18}. Similarly, we found that hydroxyaspartate, Li^+ and glial depolarization failed to affect decays of non-NMDA e.a.cs. However, non-NMDA receptors exhibit rapid desensitization¹⁹; therefore, decays of non-NMDA e.a.cs might not reflect prolongation of synaptic Glu concentrations ($[Glu]_o$) during uptake inhibition. To determine whether uptake contributes to clearance of synaptic glutamate, we used

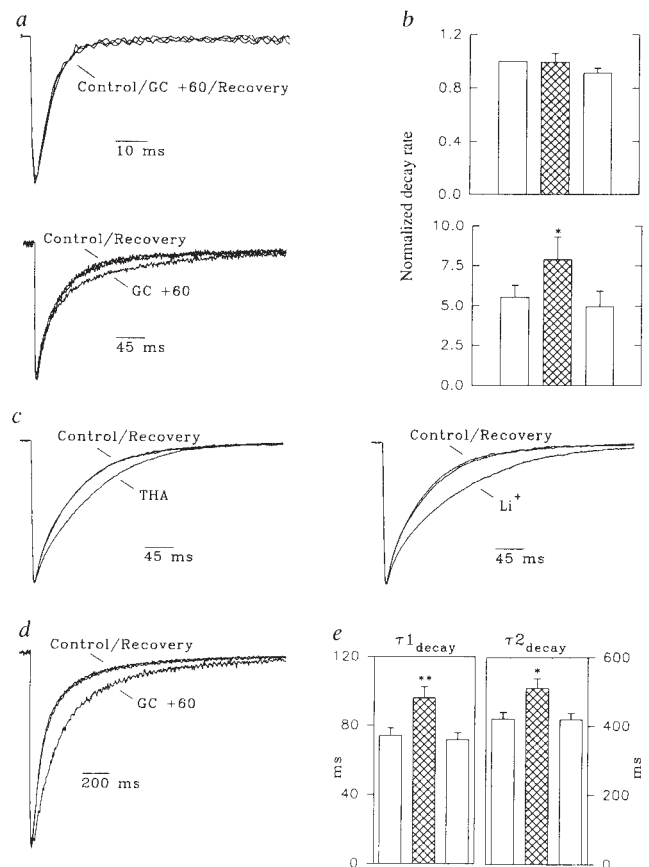


FIG. 4 Under appropriate conditions uptake inhibition prolongs e.a.cs. **a**, Top: In baseline conditions, the non-NMDA e.a.c. decay was unaffected by glial cell (GC) depolarization to $+60$ mV (GC +60) for 20 s, beginning 10 s before autaptic stimulation. Bottom: After treatment of the same micro-island with 50 μ M cyclothiazide, the decay of the non-NMDA e.a.c. was prolonged by glial depolarization. Like hydroxyaspartate and Li^+ (Fig. 2b), glial depolarization depressed e.a.cs on some micro-islands (average $<10\%$ for non-NMDA e.a.cs). To facilitate comparison of decay rates, peak currents in **a**–**d** for all conditions have been scaled to the control peak. Treatment of neurons with 0.5 μ M Glu and 50 μ M cyclothiazide depressed non-NMDA e.a.c. peaks by $12 \pm 1\%$ but did not affect e.a.c. decays ($5 \pm 3\%$ decrease in τ_{decay} , $n=5$). Therefore, Glu release from glia or tonic accumulation of Glu during uptake inhibition cannot explain the prolongation of e.a.cs during glial depolarization. **b**, Changes in e.a.c. decays for 8 pairs (recovery in cyclothiazide contains only 7 pairs) like that shown in **a**. Cyclothiazide prolonged e.a.cs and increased peak non-NMDA e.a.c. amplitudes by $54 \pm 21\%$. Often, in the presence of cyclothiazide autaptic decays were not well described by a single exponential. However, for comparison across conditions, the best single-exponential fit for each condition was normalized to the decay in the baseline (no cyclothiazide) condition. Bars represent control (left), GC +60 (cross-hatched) and recovery (right). For all graphs in this figure, $**P<0.001$, $*P<0.01$ (compared with control by paired t-test). **c**, Li^+ and hydroxyaspartate (30 μ M) also prolonged non-NMDA e.a.c. decays in the presence of cyclothiazide. The same neuron is depicted in both panels. For 8 neurons treated as in **c** in the presence of cyclothiazide, average single-exponential fits changed from 39.1 to 52.0 ms in 30 μ M hydroxyaspartate, and from 40.2 to 51.1 ms in the presence of Li^+ . For experiments in **a**–**c**, 30 μ M APV was present in all bath solutions. **d**, **e**, Glial depolarization prolongs NMDA e.a.cs. **d**, The traces show NMDA e.a.cs scaled to the control peak before, during and after depolarization of the glial cell to $+60$ mV. The external solution contained 10 μ M CNQX, 10 μ M glycine and no added Mg^{2+} or APV. Peak NMDA e.a.cs were depressed more than non-NMDA e.a.cs by uptake inhibitors ($31 \pm 4\%$ depression) during glial depolarization. This could reflect the lower IC_{50} for desensitization of NMDA receptors by Glu^{29,30}. **e**, Average changes in biexponential decays of 20 NMDA e.a.cs studied as in **d**. Bars represent control (left), GC +60 (cross-hatched) and recovery (right). There was no change in the relative amplitude of the fast component of decay ($69 \pm 1\%$, control; $68 \pm 2\%$, GC +60).

cyclothiazide to inhibit receptor desensitization^{14,20-22}. In the presence of 50 μ M cyclothiazide, which prolonged non-NMDA e.a.s by fivefold, glial depolarization, hydroxyaspartate and Li⁺ further prolonged non-NMDA e.a.s (Fig 4a-c). In 7/12 cell pairs treated with cyclothiazide, hydroxyaspartate combined with glial depolarization slowed non-NMDA e.a.c. decays more than glial depolarization alone (27 $\pm$ 5% increase in the time constant of e.a.c. decay (τ_{decay}), $n = 7$), suggesting that hydroxyaspartate-sensitive neuronal uptake also contributes to glutamate removal.

The ability of cyclothiazide to prolong e.a.s suggests that desensitization participates in evoked e.a.c. termination²⁰. However, cyclothiazide may also alter the channel closing rate, as suggested by an increase in the apparent affinity of non-NMDA receptors^{21,22}. Our data do not allow us to determine which of these changes underlies prolongation of e.a.s and the dependence of the decay on uptake. If glutamate uptake is the primary means of rapidly clearing glutamate from the synaptic cleft, there would probably be a decreasing dependence of e.a.c. decay on uptake as the apparent affinity of receptors is increased, a prediction contrary to the results obtained with cyclothiazide (Fig. 4a). However, if diffusion rapidly clears most glutamate from the cleft, and uptake removes only residual glutamate (which would not normally activate receptors), then an apparent increase in affinity might lead to greater dependence of e.a.c. decay on uptake.

Autaptic responses mediated by NMDA receptors, which exhibit higher affinity for glutamate and slower desensitization than non-NMDA receptors^{19,23}, were also prolonged by glial depolarization (Fig. 4d, e). Consistent with these results, hydroxyaspartate and Li⁺ prolonged NMDA e.a.s (THA: $\tau_{1\text{decay}}$ (the first time constant of decay of the NMDA component of e.a.s) = 75 $\pm$ 24% increase, $\tau_{2\text{decay}}$ (the second exponential time constant) = 68 $\pm$ 28% increase, $n = 5$; Li⁺: $\tau_{1\text{decay}}$ = 90 $\pm$ 23% increase, $\tau_{2\text{decay}}$ = 23 $\pm$ 16% increase, $n = 7$). These results suggest that postsynaptic receptor properties, diffusion and glutamate uptake all contribute to shaping evoked e.a.s.

Our results support emerging concepts of glia as participants in neurotransmission^{24,25}. These studies suggest that one reason for the lack of effect of uptake inhibition on non-NMDA e.p.s.c. decays (Fig. 4a and refs 17,18) is that postsynaptic receptor properties dictate the time course of non-NMDA e.p.s.c.s, despite the prolonged presence of glutamate during uptake inhibition. Further study should reveal whether the prolongation of synaptic [Glu]_o by uptake inhibition can modulate neurotransmission by altering postsynaptic receptor desensitization and/or presynaptic release²⁶. Similarly, by regulating tonic ambient glutamate levels, uptake may be important in long-term synaptic plasticity and in glutamate toxicity. □

Received 27 August; accepted 7 December 1993.

- Wyllie, D. J. A., Mathie, A., Symonds, C. J. & Cull-Candy, S. G. *J. Physiol., Lond.* **432**, 235-258 (1991).
- Barres, B. A. *J. Neurosci.* **11**, 3685-3694 (1991).
- Brew, H. & Attwell, D. *Nature* **327**, 707-709 (1987).
- Barbour, B., Brew, H. & Attwell, D. *J. Physiol., Lond.* **436**, 169-193 (1991).
- Szatkowski, M., Barbour, B. & Attwell, D. *Nature* **348**, 443-446 (1990).
- Segal, M. M. *J. Neurophysiol.* **65**, 761-770 (1991).
- Bekkens, J. M. & Stevens, C. F. *Proc. natn. Acad. Sci. U.S.A.* **88**, 7834-7838 (1991).
- Bridges, R. J., Stanley, M. S., Anderson, M. W., Cotman, C. W. & Chamberlin, A. R. *J. med. Chem.* **34**, 717-725 (1991).
- Hille, B. *J. gen. Physiol.* **59**, 637-658 (1972).
- Karwowski, C. J., Lu, H.-K. & Newman, E. A. *Science* **244**, 578-580 (1989).
- Korn, H. & Faber, D. S. *Trends Neurosci.* **14**, 439-445 (1991).
- Katz, B. & Miledi, R. *Proc. R. Soc. B161*, 496-503 (1965).
- Harrison, N. L. *J. Physiol., Lond.* **422**, 433-446 (1990).
- Zorumski, C. F., Yamada, K. A., Price, M. T. & Olney, J. W. *Neuron* **10**, 61-67 (1993).
- Clements, J. D., Lester, R. A., Tong, G., Jahr, C. E. & Westbrook, G. L. *Science* **258**, 1498-1501 (1992).
- Eccles, J. C. & Jaeger, J. C. *Proc. R. Soc. B148*, 38-56 (1957).
- Hestrin, S., Sah, P. & Nicoll, R. A. *Neuron* **5**, 247-253 (1990).
- Sarantis, M. et al. *Neuron* **11**, 541-549 (1993).
- Patneau, D. K. & Mayer, M. L. *J. Neurosci.* **10**, 2385-2399 (1990).
- Trussell, L. O., Zhang, S. & Raman, I. M. *Neuron* **10**, 1185-1196 (1993).
- Patneau, D. K., Vycklicky, L. Jr & Mayer, M. L. *J. Neurosci.* **13**, 3496-3509 (1993).
- Yamada, K. A. & Tang, C.-M. *J. Neurosci.* **13**, 3904-3915 (1993).
- Lester, R. A. & Jahr, C. E. *J. Neurosci.* **12**, 635-643 (1992).

- Cornell-Bell, A. H., Finkbeiner, S. M., Cooper, M. S. & Smith, S. J. *Science* **247**, 470-473 (1990).
- Murphy, T. H., Blatter, L. A., Wier, W. G. & Baraban, J. M. *J. Neurosci.* **13**, 2672-2679 (1993).
- Forsythe, I. D. & Clements, J. D. *J. Physiol., Lond.* **429**, 1-16 (1990).
- Lucero, M. T. & Pappone, P. A. *J. gen. Physiol.* **95**, 523-544 (1990).
- Stephens, G. J., Djamgoz, M. B. A. & Wilkin, G. P. *Receptors Channels* **1**, 39-52 (1993).
- Sather, W., Dieudonne, S., MacDonald, J. F. & Ascher, P. *J. Physiol., Lond.* **450**, 643-672 (1992).
- Colquhoun, D., Jonas, P. & Sakmann, B. *J. Physiol., Lond.* **458**, 261-287 (1992).

ACKNOWLEDGEMENTS. We thank J. Que and A. Benz for technical assistance, and R. Wilkinson, J. Huettner, J. H. Steinbach, K. Yamada, L. Thio, K. Kato, Y. Izumi and D. B. Clifford for discussion. This work was supported by grants from NIMH and the Bantky Foundation.

Understanding the controversy over the identity of EDRF

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THIRTEEN years after its discovery¹, there is still controversy over the chemical identity of endothelium-derived relaxing factor (EDRF). Although pharmacological and chemical evidence indicates that EDRF is nitric oxide², other candidates, including *S*-nitrosocysteine^{3,4}, dinitrosyl-iron-cysteine complex⁵, nitroxyl⁶ and hydroxylamine⁷, have been proposed to account for the vasorelaxant properties of EDRF. Such diverse compounds should differ in their stability and in reactivity with oxyhaemoglobin and with redox-active nucleophiles such as thiols. Here we use a bioassay to compare the pharmacodynamic profiles of these and other compounds with those of nitric oxide and EDRF. We find that some *S*-nitrosothiols, dinitrosyl-iron-cysteine complex, sodium nitroxyl and hydroxylamine can be eliminated as candidates as they are more stable than EDRF and less susceptible to inhibition by oxyhaemoglobin. Co-infusion of cysteine revealed major differences between the remaining candidates because it reduced the effect of authentic nitric oxide and EDRF on the bioassay tissues but enhanced the survival of *S*-nitrosocysteine and *S*-nitrosocysteamine. Our results further support the evidence that EDRF, the pharmacological entity described by Furchgott and Zawadzki¹, is nitric oxide.

Comparison of the relaxing actions of various EDRF candidates in a cascade superfusion bioassay system comprising three precontracted de-endothelialized rabbit aortic strips⁸ showed that nitric oxide was 3-10-fold less potent than *S*-nitrosocysteine, *S*-nitrosocysteamine and *S*-nitrosoglutathione when infused over the tissues for 1 min at equimolar concentrations (Fig. 1). The apparent greater potency of the *S*-nitrosothiols could be attributable to the breakdown of nitric oxide to inactive products, whereas *S*-nitrosothiols break down to the active nitric oxide on passage down the cascade. D- and L-*S*-nitrosocysteine were equally effective, ruling out the involvement of specific *S*-nitrosothiol receptors⁹ in the mediation of vascular relaxation. On a molar basis, hydroxylamine and sodium nitroxyl were 5-10-fold and 1,000-fold less effective, respectively, than nitric oxide. Dinitrosyl-iron-cysteine complex (DNIC) was equipotent to nitric oxide but had a considerably longer duration of action (Fig. 1), possibly because it can lodge in the tissue, as does Roussin's Black salt¹⁰. Moreover, the bioassay tissues did not recover their initial tone after addition of higher concentrations of DNIC, whereas the effect of a 30-min infusion of nitric oxide matching the potency of DNIC on the uppermost tissue was reversed when the infusion ceased (not shown).

Table 1 shows that the greater stability of DNIC, sodium nitroxyl and hydroxylamine, and their different susceptibility

FIG. 1 Comparison of the relaxing activity of authentic nitric oxide with that of various *S*-nitrosothiols, sodium nitroxy (NaNO) and hydroxylamine. The bioassay system comprised three rabbit endothelium-denuded aortic strips arranged in a cascade and superfused with oxygenated Krebs buffer (in mM: NaCl, 126.8; KCl, 5.9; MgCl₂, 1.2; NaH₂PO₄, 1.2; CaCl₂, 2.5; NaHCO₃, 30.0; glucose, 5.0; this was supplemented with 1 μ M indomethacin) at 5 ml min⁻¹. Strips were submaximally contracted with either phenylephrine (200–300 nM) or the stable thromboxane A₂ mimetic U46619 (30–50 nM) and separated from one another by a transit time of 3 s. GTN, Glyceryl trinitrate; GSNO, *S*-nitrosoglutathione; CysaNO, *S*-nitrosocysteamine; CysNO, *S*-nitrosocysteine.

METHODS. Aqueous solutions of nitric oxide were prepared as described previously¹⁹. *S*-nitrosothiols were synthesized daily by acid-catalysed *S*-nitrosation of the respective thiols with sodium nitrite at 2 °C. Isolation of solid compound was achieved by acetone precipitation, extensive washing of the collected crystals with ice-cold acetone and diethyl ether and drying in a stream of argon in the dark. Identity was verified by UV/visual spectrometry, mass spectrometry and HPLC immediately after preparation. Stock solutions in de-aerated ice-cold citrate buffer, pH 2.2, were protected from light, and all dilutions were made in argon-gassed ice-cold saline immediately before use. The vehicle alone had no effect on the bioassay. DNIC was prepared as described previously²⁰, and frozen stock solutions with a molar ratio of Fe²⁺:cysteine of 1:20 were thawed and diluted in ice-cold saline just before use. Sodium nitroxy was prepared by bubbling nitric oxide into a solution of sodium in liquid ammonia²¹ and dissolved in argon-gassed saline. Compounds were applied as 1-min infusions using gas-tight syringes. The delay time between the infusion site and the uppermost bioassay tissue was 0.5 s. A 1-min infusion of 50 nM glyceryl trinitrate served as an internal standard. Solutions were protected from light and delivered through stainless-steel tubing to prevent oxidative decomposition. The medium perfusing the assay tissues was maintained at 38 ± 0.2 °C. The half-life of each compound was estimated by comparing the maximal relaxation obtained on the first bioassay tissue with that obtained on the second and third strip, taking into account a transit time of 3 s from tissue to tissue. The molar concentration of DNIC is expressed as the concentration of nitric oxide in the complex. The tracings shown are typical of five similar experiments.

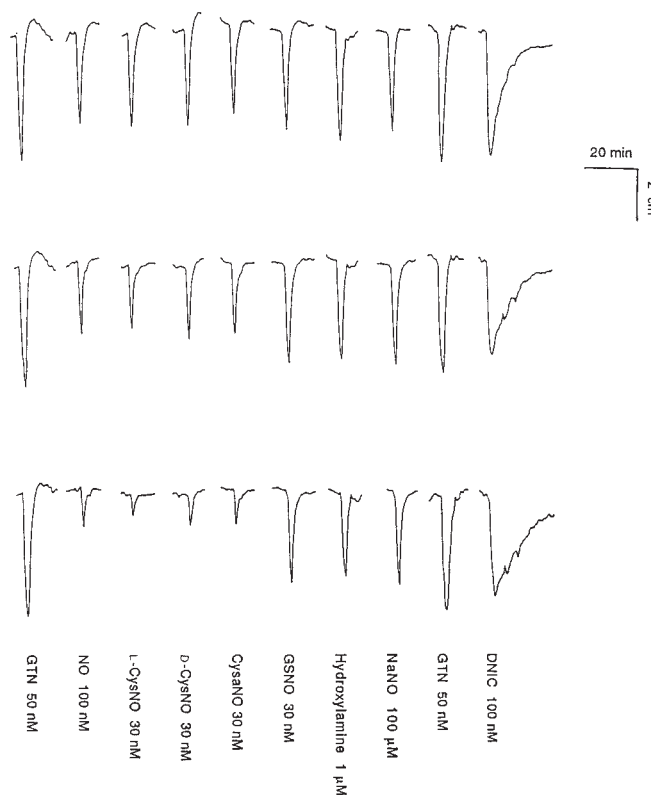


TABLE 1 Comparison of half-lives and sensitivity to inhibition by oxyhaemoglobin for some EDRF candidates

Compound	Half-life in bioassay cascade (s)	Oxyhaemoglobin IC ₅₀ (nM)
EDRF	3–5	25
Nitric oxide	3–5	25
<i>S</i> -nitrosocysteine	4–6	40
<i>S</i> -nitrosocysteamine	4–6	40
DNIC	>>EDRF*	>100
Hydroxylamine	>>EDRF*	>300
Sodium nitroxy	>>EDRF*	>300

IC₅₀, 50% inhibitory concentration.

* Half-life not measurable as there was no loss of activity during passage down the bioassay cascade.

to inhibition by oxyhaemoglobin, rule them out as potential candidates for EDRF. Other *S*-nitrosylated compounds tested (*L*-homocysteine, *N*-acetyl-DL-cysteine, glutathione, *N*-acetyl-DL-penicillamine and human serum albumin) were also considerably more stable than EDRF on passage down the cascade (not shown).

Only *S*-nitrosocysteine and *S*-nitrosocysteamine had a biological profile similar to that of nitric oxide on these bioassay tissues; however, the use of either *L*- or *D*-cysteine distinguished between the actions of these compounds. Infusion of cysteine over the detector tissues completely abrogated the relaxing effect of basally released EDRF from the rabbit aorta, as well as from endothelial cells in culture. Furthermore, cysteine inhibited the relaxing action both of authentic nitric oxide and of EDRF released by the cells following stimulation with bradykinin (Fig. 2). In contrast, the relaxing action of the *S*-nitrosothiols was unaffected on the first tissue and was markedly enhanced on the lower tissues, indicating stabilization of these compounds. This discriminatory effect of cysteine was dependent on its concentration, starting at ~1 μ M and becoming optimal at 5–10 μ M. Concentrations of cysteine higher than 100 μ M, in contrast, potentiated the effects of EDRF, nitric oxide and *S*-nitrosocysteine. Figure 2 shows the effect of a fixed concentration of *L*-cysteine (5 μ M) on equipotent concentrations of EDRF, nitric oxide, *S*-nitrosocysteine and *S*-nitrosocysteamine, so that the ratio between thiol and the active component of each EDRF candidate was constant.

The effects of increasing concentrations of *L*-cysteine on the dilator response of rat isolated aortic rings in organ baths to equipotent concentrations of nitric oxide and *S*-nitrosocysteine are shown in Fig. 3. *L*-Cysteine caused a concentration-dependent enhancement of both the magnitude and duration of the relaxation elicited by *S*-nitrosocysteine. In contrast, the action of nitric oxide was reduced at concentrations of *L*-cysteine up to

100 μ M. At higher concentrations, however, *L*-cysteine (1 mM) greatly enhanced the nitric oxide-induced relaxation, so that it was equivalent to that of *S*-nitrosocysteine in the presence of this concentration of *L*-cysteine, indicating that under these conditions ~10% of the nitric oxide applied was converted to *S*-nitrosocysteine.

Thus, the use of cysteine reveals major differences between those EDRF candidates not ruled out by potency, stability and reactivity with oxyhaemoglobin. Our data agree with a previous report that EDRF released by acetylcholine from endothelial cells behaves like nitric oxide and not like *S*-nitrosocysteine¹¹. There are various ways in which cysteine may inhibit the relaxant effects of EDRF and nitric oxide. Reaction between nitric oxide and cysteine to form *S*-nitrosocysteine¹² is an unlikely possibility as this would lead to enhanced activity on the lower bioassay tissues, which was not observed. The generation of superoxide (O₂⁻) by cysteine¹³, could account for the enhanced destruction of nitric oxide because, in isolated aortic rings in organ baths, superoxide dismutase almost completely reversed, and pyrogallol, a generator of O₂⁻ (ref 14), mimicked in a superoxide-dismutase-reversible manner the inhibitory effect of *L*-cysteine on nitric oxide-mediated relaxation (*n* = 3; not shown). Conver-

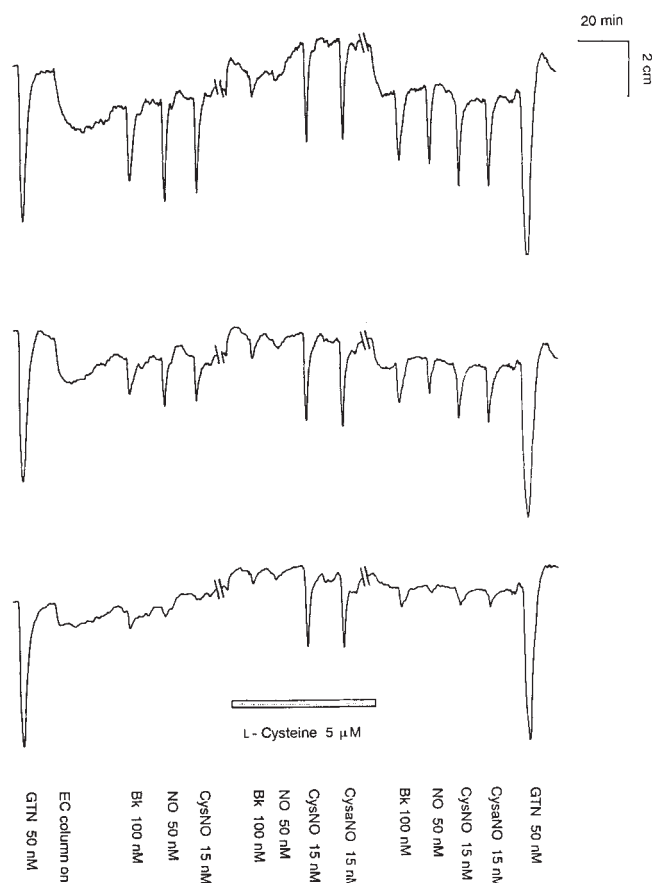


FIG. 2 The effect of L-cysteine on the relaxing actions of authentic nitric oxide, S-nitrosocysteamine (CysaNO), S-nitrosocysteine (CysNO) and EDRF released from endothelial cells in culture. Endothelial cells were isolated from porcine thoracic aorta, cultured on microcarrier beads as described previously and packed into a water-jacketed chromatography column²², the outlet of which was separated from the uppermost bioassay strip by a delay of 0.5 s. When the bioassay cascade was perfused with effluent from the column ('EC column on') there was an immediate loss of tissue tone due to basal release of EDRF. Stimulated release of EDRF from the cells was achieved by a 1-min infusion of bradykinin (Bk; 100 nM). L-Cysteine (5 μ M) was applied directly over the bioassay tissues, causing an immediate rise in tone which was reversed when the infusion was terminated. The tracing shown is representative of five experiments with similar results. EDRF released by acetylcholine (0.3 μ M) from an endothelium-intact segment of rabbit thoracic aorta gave the same bioassay profile in the presence and absence of L-cysteine as did that released from endothelial cells ($n=3$, not shown).

sion of nitric oxide to another oxidation state (NO^+ or NO^-) may occur in the presence of cysteine. Recent evidence suggests that NO^+ , as such, is not a dilator, although a small proportion may be reduced spontaneously to nitric oxide¹⁵. Although NO^- has been reported to possess potent vasodilator activity¹⁶, our results using sodium nitroxyl show that it is only relaxant at very high concentrations, suggesting that NO^- itself may not have been the species responsible for the vascular relaxation reported recently¹⁶. There is no evidence for direct chemical trapping of nitric oxide by cysteine, although this cannot be ruled out.

The enhancement of S-nitrosothiol activity by cysteine may be due to chemical stabilization. In spectrophotometric studies we found that the half-life of S-nitrosocysteine was dependent on the starting concentration of the nitrosothiol, the buffer composition and transition metal contamination of the water used. The half-life of 30 μ M S-nitrosocysteine (as estimated by the loss in absorbance at 330 nm) in 50 mM phosphate buffer, pH 7.4, at 37 $^\circ\text{C}$ was 0.31 ± 0.05 min ($n=4$). Addition of L-cysteine

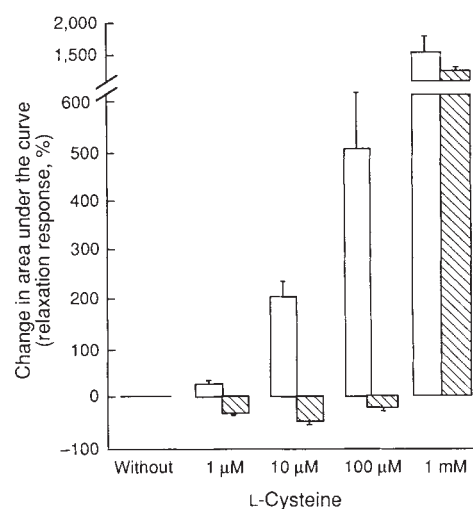


FIG. 3 Effect of different concentrations of L-cysteine on the relaxation to nitric oxide (hatched) and S-nitrosocysteine (□) of rat endothelium-intact isolated aortic rings in an organ bath. The tissues were precontracted with 200 nM phenylephrine and the response to equipotent concentrations of nitric oxide (3 μ M) and S-nitrosocysteine (300 nM) was determined in the presence of increasing concentrations of L-cysteine added to the organ bath 5 min before addition of either compound. A single concentration of L-cysteine was used for each tissue and its effects on both vasodilators were compared. At concentrations of L-cysteine between 1 μ M and 100 μ M there was a marked difference in its effect on nitric oxide and S-nitrosocysteine mediated relaxation. However, at 1 mM, L-cysteine potentiated the relaxant effects of both compounds. Results shown are means $\pm$ s.e.m. of three separate experiments.

(10 μ M–100 mM) increased the stability of S-nitrosocysteine in a concentration-dependent manner, so that, at a molar ratio of S-nitrosocysteine to L-cysteine identical to that in the cascade experiments (1:333), the half-life of 30 μ M S-nitrosocysteine was 36.9 ± 3.7 min ($n=4$). EGTA and EDTA mimicked the effects of L-cysteine, suggesting that the stabilization of S-nitrosocysteine is probably due to the ability of thiols to complex metal ions present in the buffer solution, assuming that metal ion catalysis is the predominant pathway for the release of nitric oxide from S-nitrosothiols¹⁷.

We suggest that the discriminatory effect of cysteine on nitric oxide and S-nitrosothiols is due to its ability both to generate O_2^- and to complex metal ions. Its -SH moiety probably confers these properties, as we observed similar effects with L-homocysteine, glutathione and human serum albumin (not shown). Because free sulphhydryl groups can also react with oxidized forms of nitric oxide, this explains the paradoxical effect of thiols, as at low concentrations they will act as inactivators and at high concentrations, via S-nitrosation, as carriers for nitric oxide. The physiological concentration of thiols is of the order of 0.1–1 mM¹⁸. In all classical bioassay systems for EDRF, thiol availability is limited, and this, as is known for oxygen concentration, has a bearing on the pharmacological half-life of EDRF. Moreover, the half-life and fate of nitric oxide depend on its concentration and the fact that physiological concentrations of nitric oxide are in the nanomolar range will limit its reactivity with molecules such as amines, thiols and O_2^- .

Therefore, we would like to re-state that EDRF, as described by Furchgott and Zawadzki¹, is nitric oxide. Furthermore, the molecule responsible for transmitting the biological information provided by the L-arginine–nitric oxide pathway is most probably also nitric oxide; however, any claims about physiology will require a clearer understanding of the reactivity of nitric oxide at low concentrations in physiological environments. □

Received 12 August; accepted 21 December 1993.

1. Furchgott, R. F. & Zawadzki, J. V. *Nature* **288**, 373–376 (1980).

2. Palmer, R. M. J., Ferrige, A. G. & Moncada, S. *Nature* **327**, 524–526 (1987).
3. Myers, P. R., Minor, R. L. Jr, Guerra, R. Jr, Bates, J. & Harrison, D. G. *Nature* **345**, 161–163 (1990).
4. Rubanyi, G. M., Johns, A., Harrison, D. & Wilcox, D. *Circulation* (suppl.) **80**, II-281 (abstr.) (1988).
5. Vanin, A. F. *FEBS LETT.* **289**, 1–3 (1991).
6. Fukuto, J. M., Hsieh, R. & Chiang, K. T. *FASEB J.* **6**, A972 (abstr.) (1992).
7. Thomas, G. & Ramwell, P. W. *Biochem. biophys. Res. Commun.* **164**, 889–893 (1989).
8. Palmer, R. M. J., Ferrige, A. G. & Moncada, S. *Nature* **327**, 524–526 (1987).
9. Lewis, S. J. et al. *FASEB J.* **6**, A1165 (abstr.) (1992).
10. Flitney, F. W., Megson, I. L., Flitney, D. E. & Butler, A. R. *Br. J. Pharmac.* **107**, 842–848 (1992).
11. Furchgott, R. F., Jothianandan, D. & Khan, M. T. *Jpn. J. Pharmac.* **58**, (Suppl. 2), 185P–191P (1992).
12. Stamler, J. S. et al. *Proc. natn. Acad. Sci. U.S.A.* **89**, 444–448 (1992).
13. Misra, H. P. *J. biol. Chem.* **269**, 2151–2155 (1974).
14. Moncada, S., Palmer, R. M. J. & Gryglewski, R. J. *Proc. natn. Acad. Sci. U.S.A.* **83**, 9164–9168 (1986).
15. Zamora Pino, R. & Feilisch, M. *Endothelium* **1**, S69 (abstr.) (1993).
16. Fukuto, J. M., Chiang, K., Hsieh, R., Wong, P. & Chaudhuri, G. *J. Pharmac. exp. Ther.* **263**, 546–551 (1992).
17. McAninly, J., Williams, D. L. H., Askew, S. C., Butler, A. R. & Russell, C. *JCS chem. Commun.* **23**, 1758–1759 (1993).
18. Jocelyn, P. C. *Biochemistry of the SH Group* (Academic, New York, 1972).
19. Feilisch, M. *J. cardiovasc. Pharmac.* **17**, S25–S33 (1991).
20. Vedernikov, Y. P., Mordvintsev, P. I., Maichenkova, I. V. & Vanin, A. F. *Eur. J. Pharmac.* **221**, 313–317 (1992).
21. Zintl, E. & Harder, A. *Chem. Ber.* **66**, 760–761 (1933).
22. Kelm, M. et al. *Biochem. biophys. Res. Commun.* **154**, 236–244 (1988).

ACKNOWLEDGEMENTS. We thank A. F. Vanin for DNIC, D. Rees and Y.-C. Su for technical assistance, and A. Higgs for help in preparing the manuscript.

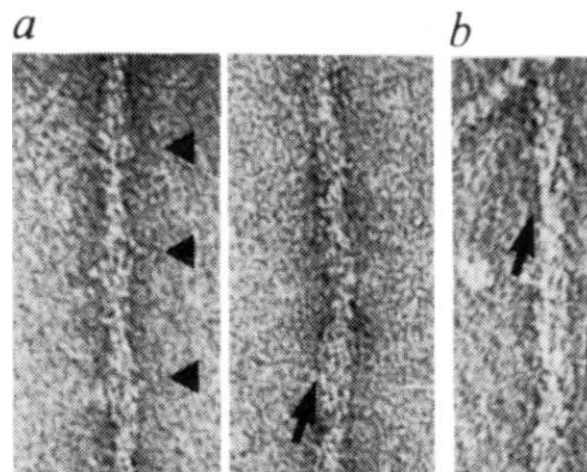


FIG. 1 Electron micrographs of *Limulus* thin filaments negatively stained with uranyl acetate: a, in EGTA, and b, in Ca^{2+} ; bulges repeating at 40-nm intervals (arrowheads) and narrow helically oriented strands (arrows) are indicated. Scale bar, 0.05 μm . ATPase was assayed^{7,8} on three preparations of thin filaments isolated^{5,6} for electron microscopy. Each fully activated rabbit skeletal muscle myosin (ATPase activity, $0.60 \pm 0.04 \mu\text{mol min}^{-1}$ per mg myosin); calcium sensitivity (per cent inhibition of ATPase on removal of Ca^{2+}) was $92 \pm 1\%$. Ca^{2+} sensitivity was unaffected by dilution to the concentrations (0.03 – 0.1 mg ml^{-1}) necessary for microscopy and was stable for several months, although only freshly prepared samples were used for negative staining²¹. Images were recorded using the Minimal Dose System of a JEOL 100CX electron microscope²¹ at a magnification of $\times 50,000$ and digitized on an Eikonix 1412 scanner for image analysis¹¹.

Ca^{2+} -induced tropomyosin movement in *Limulus* thin filaments revealed by three-dimensional reconstruction

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THE steric model of muscle regulation holds that tropomyosin strands running along thin filaments move away from myosin-binding sites on actin when muscle is activated. Exposing these sites would permit actomyosin interaction and contraction to proceed. This compelling and widely cited model is based on changes observed in X-ray diffraction patterns of skeletal muscle following activation^{1–3}. Although analysis of X-ray patterns can suggest models of filament structure, unambiguous interpretation is not possible. In contrast, three-dimensional reconstruction of thin-filament electron micrographs could, in principle, offer direct confirmation of the predicted tropomyosin movement, but so far tropomyosin in skeletal muscle has been resolved definitively only in the 'on' state but not in the 'off' state⁴. Thin filaments from the arthropod *Limulus* have a similar composition to those from vertebrate skeletal muscle⁵, and troponin–tropomyosin is distributed in both species with the same characteristic 38-nm periodicity⁶. *Limulus* thin filaments activate skeletal muscle myosin ATPase at micromolar Ca^{2+} concentrations and confer a high calcium dependence on the enzyme. Arthropod and vertebrate troponin subunits form functional hybrids *in vitro*⁷ and the respective tropomyosins are functionally interchangeable^{8,9}, arguing for a common mechanism of thin-filament-linked regulation in the two phyla. Here we report that tropomyosin is readily resolved in native filaments of troponin-regulated *Limulus* muscle in both the 'on' and 'off' states, and demonstrate tropomyosin movement, providing support for the importance of steric effects in muscle activation.

Negatively stained *Limulus* thin filaments (Fig. 1) isolated in EGTA and either maintained in EGTA or treated with Ca^{2+} show the characteristic double-helical array of actin subunits and in addition frequently display periodic bulges repeating at 40-nm intervals (compare ref. 10). These bulges are presumably a manifestation of the large mass of *Limulus* troponin⁵ as they are not obvious in vertebrate filaments in which troponin has smaller mass. *Limulus* filaments also often exhibit elongated strands aligned with the long-pitch actin helices, which we have previously shown¹¹ to be a reflection, at least in part, of extended tropomyosin molecules. In any given field, however, strands and periodic bulges are not always evident, possibly because of varied troponin subunit orientation or stain penetration.

Density maps (Figs 2, 3) calculated from the averaged layer-line data of ten filaments maintained in EGTA (Fig. 4) reveal actin monomers whose bi-lobed, two-domain shape and monomer–monomer connectivity are indistinguishable from those in reconstructions of negatively stained vertebrate smooth muscle thin filaments¹¹ and frozen hydrated skeletal filaments⁴. In addition, a longitudinally continuous strand of density follows successive actin monomers in contact with the extreme inner edge of their outer domains.

Density maps (Figs 2, 3) calculated from the averaged layer line data of six Ca^{2+} -treated filaments (Fig. 4) reveal actin monomer shape and connectivity very similar to those seen in the EGTA-treated filaments. The elongated strand of density, however, is now located in a different position, closely associated with the inner domain of actin. Comparison of the EGTA and Ca^{2+} maps shows that the strand has moved azimuthally by $\sim 25^\circ$ about the actin helix axis. Statistical tests demonstrate that the Ca^{2+} -induced strand rotation is significant at greater than the 99.5% confidence level. Although there is partial overlap in the strand position, the point of contact with actin is distinct in the two maps.

Tropomyosin, an elongated molecule¹², is likely to make the major contribution to the strand observed in *Limulus* thin filaments. Troponin subunit T in vertebrates is also an asymmetric molecule that binds to tropomyosin¹³, and, by analogy, *Limulus*

FIG. 2 Surface views of reconstructed densities of thin filaments (a, c) in EGTA and (b) in Ca^{2+} . Two density levels are presented: in a and b a lower level shows the helically wound strands on the surface of actin, contoured to give strands a diameter of about 20 Å, the dimensions of tropomyosin; in c, a higher level shows the point of contact of the strand with actin monomers in EGTA. The inner (A_i) and outer (A_o) domains of one actin monomer are marked. Note the different position of the strands running along A_o in a and c, and along A_i in b. At the contour level chosen in c, it becomes evident that in EGTA the strands make contact with the extreme inner edge of A_o , whereas in Ca^{2+} (b) they do not make contact with A_o . Scale bar, 100 Å. Helical reconstruction was by standard procedures^{22,23} as previously¹¹. When data are omitted very close to the meridian ($R < 0.012 \text{ Å}^{-1}$) on the $l=1$, $l=2$ and $l=3$ layer lines (Fig. 4) that arise from the axial repeat of troponin (and reflect most of the scattering from the globular parts of troponin), there is no impact on the bi-lobed form of actin or the appearance or position of the strand; similarly, truncating micrographs at the lateral edges of thin-filament images to remove troponin contribution has little impact on subsequent reconstruction. It is therefore unlikely that the 'troponin bulges' *per se* are responsible for the strands observed, but rather that elongated tropomyosin molecules are the major determinant.

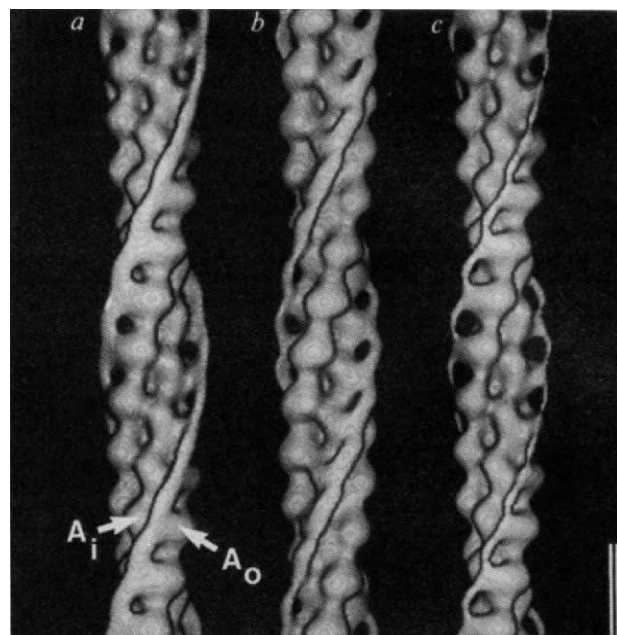


FIG. 3 Helical projections formed by projecting densities in our maps along their $n=2$ helical tracks onto a plane perpendicular to the helix axis. a, Thin filaments in EGTA; b, thin filaments in Ca^{2+} . The tropomyosin strand, which makes contact with actin monomers at A_o and A_i (see Fig. 2) in a and b respectively, is indicated by arrows. The statistical significance of the difference between the two maps was computed by point-by-point comparison^{24,25}; a difference map (not shown) was calculated by subtracting densities in the Ca^{2+} map from those in the EGTA map, and the significance of the difference evaluated by using a Student's *t*-test. c, The regions of the maps that are significantly different at $>99.5\%$ confidence level (white, significant 'positive' differences; black, significant 'negative' differences); the pairs of positive and negative peaks are located at the respective strand positions and demonstrate the significance of strand movement.

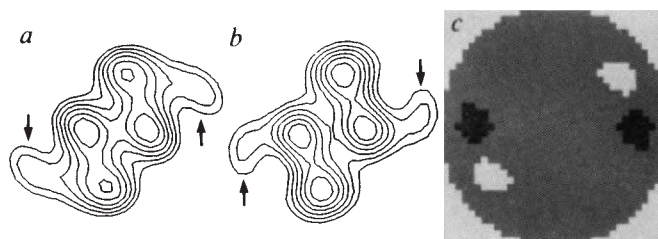
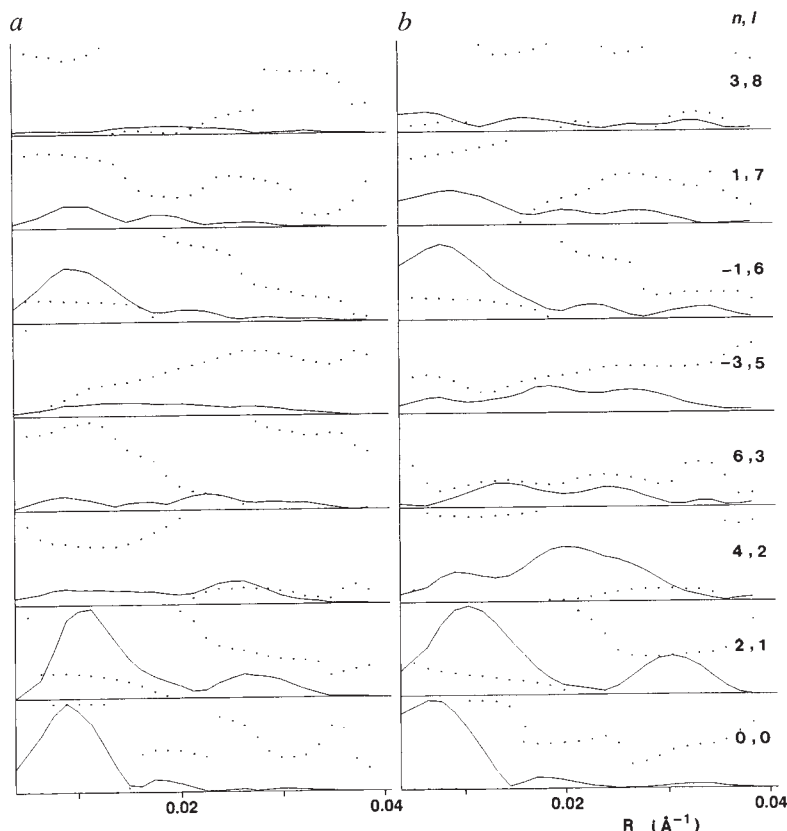


FIG. 4 The averaged layer-line data derived from Fourier transforms of a, ten thin filaments in EGTA, and b, six in Ca^{2+} ; amplitudes are represented as solid lines and phases as dotted lines. Rotational (Bessel) orders (n) and layer line numbers (l) allowed by the 13/6 actin-helical symmetry imposed on the data are shown. Fourier analysis was by standard procedures^{11,22,23}. EGTA-treated filaments were selected for averaging after (1) computing a preliminary average of five filaments which showed clear bi-lobed actin and good phase agreement with previously published thin filament maps; (2) using this average as a reference to select from among the near- and far-side datasets of 35 bulgy filaments those with the best relative fit based on their phase agreement. The layer lines used to compute a final density map were generated by averaging the Fourier terms of twelve such datasets derived from ten filaments, producing layer-line data that extend to a resolution of $1/25 \cdot 30 \text{ Å}$ radially and $\sim 1/45 \text{ Å}$ axially. Six Ca^{2+} -treated filaments were selected for averaging from an original set of 29 filaments using the same process. Filaments in either the EGTA or the Ca^{2+} series were aligned for averaging by rotation and translation, minimizing phase residuals (ψ) between layer-line datasets of individual filament sides and a preliminary average of these sides²³. In the EGTA series, ψ was $53.7 \pm 4.5^\circ$, and in Ca^{2+} was $51.3 \pm 4.2^\circ$. The up-down phase residual ($\Delta\psi$), a measure of filament polarity, was $26.2 \pm 7.6^\circ$ for the EGTA series and $24.2 \pm 7.3^\circ$ for Ca^{2+} . Note the magnitudes of peaks along the second layer line ($l=2$) are larger in the pattern associated with the Ca^{2+} -treated than the EGTA-treated filaments. This change presumably reflects enhancement of the structure's 4-fold symmetry and agrees with results of X-ray diffraction on intact muscle¹⁻³.



troponin T or other extended parts of the *Limulus* troponin complex might also contribute to the strand density. By localizing *Limulus* tropomyosin (and possibly parts of troponin), in both the 'off' and 'on' states, to positions on actin originally proposed in the seminal steric model^{1,3} and again more recently on the basis of further modelling^{14,15}, we offer direct structural support for the hypothesis. Earlier reconstructions of thin filaments reconstituted from vertebrate proteins either failed to resolve tropomyosin from actin^{16,17}, or revealed it only in the 'on' state^{4,18}, perhaps because in the vertebrate 'off' state tropomyosin is disordered¹⁸ (R. A. Milligan, personal communication).

It is striking that the position adopted by *Limulus* tropomyosin in the 'off' state coincides with a myosin contact site on actin thought to be involved in a stereospecific and strong actomyosin interaction¹⁹. Hence tropomyosin in the 'off' state may compete for myosin attachment to this site. Our results therefore are consistent with a steric mechanism in which tropomyosin in relaxed muscle could interfere with the transition from an initially weak to a strong myosin crossbridge association on actin (compare ref. 20), thereby inhibiting ATPase and crossbridge cycling. Such a mechanism is no doubt a general feature of troponin-regulated muscles. □

Received 25 November 1993; accepted 18 January 1994.

- Huxley, H. E. *Cold Spring Harbor Symp. quant. Biol.* **37**, 361–376 (1972).
- Haselgrove, J. C. *Cold Spring Harbor Symp. quant. Biol.* **37**, 225–234 (1972).
- Parry, D. A. D. & Squire, J. M. *J. molec. Biol.* **75**, 33–55 (1973).
- Milligan, R. A., Whittaker, M. & Safer, D. *Nature* **348**, 217–221 (1990).
- Lehman, W., Regenstein, J. M. & Ransom, A. L. *Biochim. biophys. Acta* **434**, 215–222 (1976).
- Lehman, W. *J. molec. Biol.* **154**, 385–391 (1982).
- Lehman, W. *Nature* **255**, 424–426 (1975).
- Lehman, W. & Szent-Györgyi, A. G. *J. gen. Physiol.* **59**, 375–387 (1975).
- Regenstein, J. M. & Szent-Györgyi, A. G. *Biochemistry* **14**, 917–925 (1975).
- Bullard, B. et al. *J. molec. Biol.* **204**, 621–637 (1988).
- Vibert, P., Craig, R. & Lehman, W. *J. Cell Biol.* **123**, 313–321 (1993).
- Cohen, C. et al. *Cold Spring Harbor Symp. quant. Biol.* **37**, 287–297 (1972).
- Flicker, P. F., Phillips, G. N. & Cohen, C. *J. molec. Biol.* **162**, 495–501 (1982).
- Holmes, K. C. & Kabsch, W. *Curr. Opin. Struct. Biol.* **1**, 270–280 (1991).
- Squire, J. M., Al-Hayat, H. A. & Yagi, N. *J. chem. Soc. Farad. Trans.* **89**, 2717–2726 (1993).

- Seymour, J. & O'Brien, E. J. *Nature* **283**, 680–682 (1980).
- Toyoshima, C. & Wakabayashi, T. *J. Biochem. (Tokyo)* **97**, 245–263 (1985).
- O'Brien, E. J., Couch, J., Johnson, G. R. P. & Morris, E. P. in *Actin: Structure and Function in Muscle and Non-Muscle Cells* (eds dos Remedios, C. G. & Barden, J. A.) 3–15 (Academic, North Ryde, NSW, Australia, 1983).
- Rayment, I. et al. *Science* **261**, 58–65 (1993).
- Chalovich, J. M., Chock, P. B. & Eisenberg, E. *J. biol. Chem.* **256**, 575–587 (1981).
- Moody, C., Lehman, W. & Craig, R. *J. Muscle Res. Cell Motil.* **11**, 176–185 (1990).
- DeRosier, D. J. & Moore, P. B. *J. molec. Biol.* **52**, 355–369 (1970).
- Amos, L. A. & Klug, A. *J. molec. Biol.* **99**, 51–73 (1975).
- Trachtenberg, S. & DeRosier, D. J. *J. molec. Biol.* **195**, 581–601 (1987).
- Milligan, R. A. & Flicker, P. F. *J. Cell Biol.* **105**, 29–39 (1987).

ACKNOWLEDGEMENTS. We thank C. Cohen for discussion and encouragement, A. McGough, D. Morgan and C. Owen for discussion and help with computer programs, and M. Picard Craig for photography. This work was supported by grants from the NIH (W.L., R.C. and C.C.) and from the NSF (P.V. and C.C.).

Calcium channel β -subunit binds to a conserved motif in the I–II cytoplasmic linker of the α_1 -subunit

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THE β -subunit is an integral component of purified voltage-sensitive Ca^{2+} channels^{1–3}. Modulation of Ca^{2+} channel activity by the β -subunit, which includes significant increases in transmembrane current and/or changes in kinetics, is observed on coexpression of six α_1 -subunit genes with four β -subunit genes in all α_1 – β combinations tested^{4–12}. Recent reports suggest that this regulation is not due to targeting of the α_1 -subunit to the plasma membrane but is probably a result of a conformational change induced by the β -subunit^{11,13}. Here we report that the β -subunit binds to the cytoplasmic linker between repeats I and II of the dihydropyridine-sensitive α_1 -subunits from skeletal (α_{1S}) and cardiac muscles (α_{1C-A}), and also with the more distantly related neuronal α_{1A} and ω -conotoxin GVIA-sensitive α_{1B} -subunits. Sequence analysis of the β -subunit binding site identifies a conserved motif (QQ-E–L–GY–WI–E) positioned 24 amino acids from the IS6 transmembrane domain in each α_1 -subunit. Mutations within this motif

reduce the stimulation of peak currents by the β -subunit and alter inactivation kinetics and voltage-dependence of activation. Conservation of the β -subunit binding motif in these functionally distinct calcium channels suggests a critical role for the I–II cytoplasmic linker of the α_1 -subunit in channel modulation by the β -subunit.

An ³⁵S-labelled *in vitro*-translated rat β_{1B} -subunit¹⁴ protein probe (Fig. 1a) was used to demonstrate an interaction between the β - and α_{1S} -subunits of the dihydropyridine receptor (DHPR) from skeletal muscle. A single prominent radioactive band corresponding to a relative molecular mass of 170,000 (M_r 170K) was detected in purified DHPR¹⁵ which colocalized with immunostained α_{1S} -subunit (Fig. 1b). On prolonged exposure, β -subunit interaction with the DHPR α_{1S} -subunit was also detected in triads and T-tubule membranes. Incubations with ³⁵S-labelled sham *in vitro* translates showed no interaction (not shown). In addition, the *in vitro*-translated β_{1A} -subunit of DHPR and the β_3 -subunit of the ω -conotoxin GVIA receptor (CgTxR)³ identified the same 170K protein, suggesting a conserved site for α_1 -subunit interaction among β -subunits from different genes (not shown).

To identify the β -subunit interaction site on the α_{1S} -subunit, we screened an epitope library of the rabbit DHPR α_{1S} -subunit¹⁶ with the ³⁵S-labelled β_{1B} -subunit probe. Figure 2a shows an autoradiogram of a single purified positive clone amplified and probed with the translated β_{1B} -subunit. Similar results were obtained with a β_3 -probe (not shown), whereas an overlay of this α_{1S} -positive clone with a ³⁵S-labelled α_{2B} -probe showed no interaction (Fig. 2a). Seven positive plaques were purified to homogeneity and DNA sequencing showed that all clones were in the appropriate reading frame. The epitopes ranged in size from 50 to 67 amino acids and all shared a 45-amino-acid overlap that extends from amino acid 341 to 385 of the α_{1S} -subunit. Analysis of the transmembrane topology of the α_{1S} -subunit maps this β -subunit interaction site to the putative cytoplasmic linker between repeats I and II and is consistent with the predicted cytoplasmic location of the β -subunit^{1,2} (Fig. 2d).

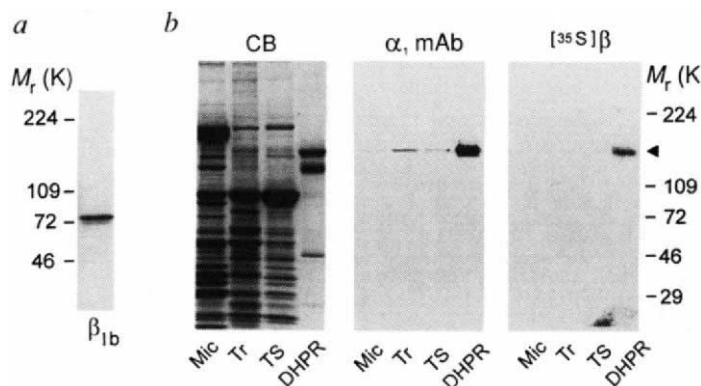
Because coexpression of β -subunits with various α_1 -subunits greatly enhances peak inward currents and alters kinetic and voltage-dependent properties^{4,12}, we addressed whether this interaction site is conserved in distantly related calcium channel α_1 -subunits. We constructed epitope libraries of the complemen-

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tary DNA encoding the rabbit cardiac dihydropyridine-sensitive α_{1C-a} -subunit⁵ and the rat neuronal ω -conotoxin GVIA-sensitive α_{1B} -subunit¹⁷. Although these subunits exhibit the characteristic transmembrane topology of voltage-sensitive calcium channels, they are encoded by different genes and share only 66 and 34% identity, respectively, with the DHPR α_{1S} . Positive clones were again detected with ³⁵S-labelled β_{1b} -subunit probe and found to map to the same location on the I-II cytoplasmic linker of each

α_1 -subunit. The smallest of two overlapping clones from the α_{1C-a} λ gt11 expression library encoded an 84-amino-acid epitope extending from amino acids 434 to 517 of this subunit. Similarly, the smallest of two epitopes cloned from a screen of the α_{1B} library yielded a 56-amino-acid domain representing amino acids 378 to 434. To extend these observations to the α_{1A} -subunit, which shares 33% identity with DHPR α_{1S} , we constructed a glutathione-S-transferase (GST) fusion protein expressing

FIG. 1 β -Subunit interaction with the α_1 -subunit of the dihydropyridine-sensitive calcium channel from skeletal muscle. **a**, Autoradiogram of SDS-polyacrylamide gel of *in vitro*-translated ³⁵S-labelled probe. The β_{1b} -subunit migrates differently from the β_{1a} of DHPR because the primary structure of the β_{1b} splice variant predicts it to have an M_r 7.81K larger than β_{1a} ¹⁴. **b**, CB, Coomassie blue-stained SDS-polyacrylamide gel of rabbit skeletal muscle crude membranes (Mic), triads (Tr), T-tubule system (TS) and purified dihydropyridine receptor (DHPR). α_1 mAb, Corresponding immunoblot stained with a mix of monoclonal antibodies (mAbs IIF7, IIC12 and IIC15; ref. 15) to α_{1S} of DHPR. [³⁵S] β , Autoradiogram of an identical nitrocellulose transfer incubated with *in vitro*-translated [³⁵S] β_{1b} probe. Arrowhead indicates the 170K protein, α_{1S} . METHODS. The [³⁵S] β_{1b} -subunit¹⁴ probe was synthesized by coupled *in vitro* transcription and translation in the TNT system (Promega). To enhance probe yield, a 50-nucleotide alfalfa mosaic viral consensus initiation site was engineered into the cDNA. Translation was in the presence of a cocktail containing protease inhibitors pepstatin A, chymostatin, aprotinin, antipain and leupeptin (Boehringer Mannheim) at 0.1 μ g ml⁻¹ and calf liver tRNA (Sigma) at 40 μ g ml⁻¹ to minimize proteolysis and reduce background translation. Rabbit skeletal muscle crude membranes, triads, T-tubule system and purified dihydropyridine receptor¹⁵ were electrophoretically separated on 3–12% SDS polyacrylamide gels in the presence of 1% 2-mercaptoethanol and transferred



to nitrocellulose. These blots were blocked with 5% non-fat dry milk in 150 mM NaCl, 50 mM sodium phosphate (PBS), followed by an overlay buffer of 5% bovine serum albumin (BSA) and 0.5% non-fat dry milk in PBS. The translation reaction was added at 1 μ l ml⁻¹ of overlay buffer and incubated with gentle mixing for 12 h at 4 °C. The transfers were washed 1 h with 5% BSA in PBS at room temperature, air-dried and exposed to film (X-OMAT AR, Kodak).

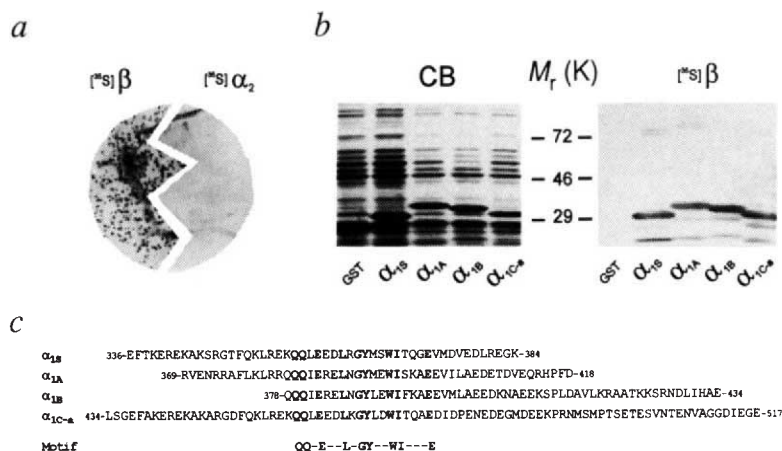


FIG. 2 Identification of the β -subunit interaction site on the α_1 -subunit by epitope cloning. **a**, Autoradiogram of an amplified single positive plaque isolated by screening a skeletal muscle DHPR α_{1S} subunit epitope library with an *in vitro*-translated [³⁵S] β_{1b} subunit probe. The nitrocellulose lift was cut and each half was incubated with either the [³⁵S] β_{1b} subunit or a [³⁵S] β_{2b} subunit probe. **b**, Coomassie blue-stained SDS-polyacrylamide gel of glutathione-S-transferase (GST) control and α_{1S} , α_{1A} , α_{1B} and α_{1C-a} epitopes expressed as GST fusion proteins in total *E. coli* lysate (left), and autoradiogram of corresponding overlay with [³⁵S] β_{1b} subunit on these nitrocellulose-immobilized fusion proteins (right). **c**, Alignment of amino-acid sequences of these fusion protein epitopes identifying a conserved α_1 - β subunit interaction motif. The first and last amino acids of each epitope are numbered according to their location in the primary structure, as deduced from the full-length cDNA. **d**, Schematic showing the β -subunit interaction site on the I-II cytoplasmic linker of the α_1 -subunit.

METHODS. α_1 -Subunit epitope libraries in the λ gt11 vector were made by digesting the α_1 -subunit cDNA in a plasmid vector with DNase I in the presence of 10 mM MnCl₂. Following the addition of EcoRI linkers, the randomly digested fragments were size-selected to <500 bp in Nusieve agarose gel (FMC Bioproducts). These fragments were then purified, digested with EcoRI and ligated into λ gt11. The β_{1b} -subunit

probe was used to screen 2×10^4 clones of each α_1 -subunit epitope library. Inserts were amplified from pure phage positives by polymerase chain reaction (PCR) using primers directed to λ gt11 phage arms. These were directly subcloned into a T-vector²³ (made from Bluescript SK-plasmid) for sequencing, or digested with EcoRI and ligated into the pGEX1 vector for GST fusion protein production. A fusion protein epitope of the α_{1A} subunit was constructed by amplifying base pairs 1,387–1,553 with the following primers: 5'-AGGGAATTCAGGGATGTCTCTG-3' and 5'-AAGGATCCGAGCGGTGGAGAAC-3'. This PCR product was then digested with BamHI and EcoRI and subcloned into the pGEX2T vector. All inserts were sequenced in both directions by the dideoxy chain termination method using Sequenase II (US Biochemicals). Each recombinant pGEX molecule was introduced into *E. coli* DH5 α cells. Overnight cultures of the pGEX-epitope constructs were diluted 1:10, incubated for 1 h and induced for 2 h with 1 mM isopropyl- β -D-thiogalactopyranoside. 75 μ l of each culture was dissolved in SDS sample buffer and proteins separated electrophoretically on 3–12% SDS-polyacrylamide gels and transferred to nitrocellulose. Overlays were done as described in the legend to Fig. 1 in 5% BSA and 0.5% non-fat dry milk in PBS. Peptide sequence homology searches were done at the National Center for Biotechnology Information using the BLAST¹⁸ network service.

amino acids 369 to 418 of the rabbit neuronal α_{1A} -subunit¹², which spans the region common to all cloned epitopes. As shown in Fig. 2b, the β_{1B} -subunit binds to the epitopes in all four α_1 -subunits when expressed as GST fusion proteins. All epitopes mapped to the I–II cytoplasmic linker, although a comparison of this linker sequence among the four α_1 -subunits shows only 19% overall identity.

Sequence comparison of the epitopes (Fig. 2c) suggests the presence of an interaction motif on each α_1 -subunit that can be minimally described by QQ-E--L-GY--WI---E. A BLAST¹⁸ search with this motif identified sequence records representing all voltage-sensitive calcium channel α_1 -subunits so far reported. Although the lengths of the cytoplasmic linkers separating repeats I and II among these four α_1 -subunits vary in size from 99 to 129 amino acids, the conserved motif is always positioned 24 amino acids downstream from the S6 transmembrane element of repeat I. Splice variations in the I–II cytoplasmic linker encoded by the α_{1C} gene have been reported which are close to the motif and hence may be important in differential β -subunit regulation of these channels. These include a 33-amino-acid exon that replaces the IS6 transmembrane segment and a 25-amino-acid insertion positioned 19 residues downstream of the motif in the α_{1C} cloned from lung¹⁹. Three additional splice variations of this region in α_{1C} have been reported at a location immediately downstream of the latter insertion²⁰.

To perturb the β -subunit binding in the protein overlay assay and thereby identify candidate residues that contribute to the structural integrity of the motif, we made the following non-conservative mutations in the GST fusion protein (FP) epitope

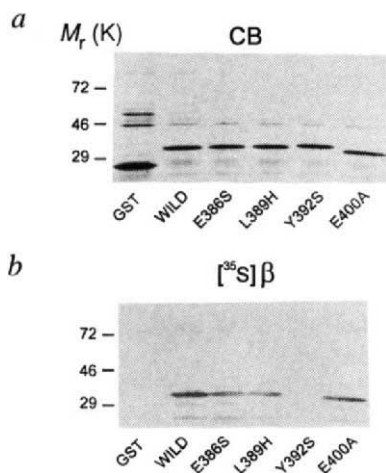


FIG. 3 Mutation of the motif in the α_{1A} epitope perturbs the β -subunit interaction in the overlay assay. **a**, Coomassie blue-stained SDS-polyacrylamide gel of GST, α_{1A} epitope (WILD) and mutants (E386S, L389H, Y392S and E400A) expressed as GST fusion proteins in total *E. coli* lysate, and **b**, autoradiogram of corresponding overlay with $[^{35}\text{S}]\beta_{1B}$ -subunit probe on these nitrocellulose-immobilized GST fusion proteins. **METHODS.** Site-directed mutagenesis was performed on the fusion protein containing the α_{1A} epitope using the Transformer site-directed mutagenesis system (Clontech). This method works by simultaneously annealing two oligonucleotide primers to one strand of a denatured double-stranded plasmid. One primer introduces the desired mutation and the second mutates a restriction site unique to the plasmid for the purpose of selection. To generate fusion protein mutants the following mutagenic primers were used: 5'-GCGGCAGCAGCAGATATCAGC-GGAGCTCAACGGG-3' (E386S), 5'-GCAGATTGAACGCGAGCACAAAC-GGGTACATGGAG-3' (L389H), 5'-CGAGCTCAACGGGTCCATGGAGTGG-ATCTCAAAAGC-3' (Y392S) and 5'-GGATCTCAAAAGCAGCTGAGG-TGATCCTCGCAGAGG-3' (E400A). The selection primer 5'-GACATCC-CITGGATATCATCGTGAAGT-3' was used to mutate *EcoRI* to *EcoRV* on the recombinant PGEX2T molecule. All mutations were verified by sequence analysis. Overlays were done as described in Fig. 1 legend. Autoradiographs were scanned with a Molecular Dynamics computing densitometer.

of the α_{1A} -subunit: glutamate at position 4 of the motif to serine (E386S_{FP}), leucine at position 7 to histidine (L389H_{FP}), tyrosine at position 10 to serine (Y392S_{FP}) or glutamate at position 18 to alanine (E400A_{FP}). The ability of the mutants to interact with

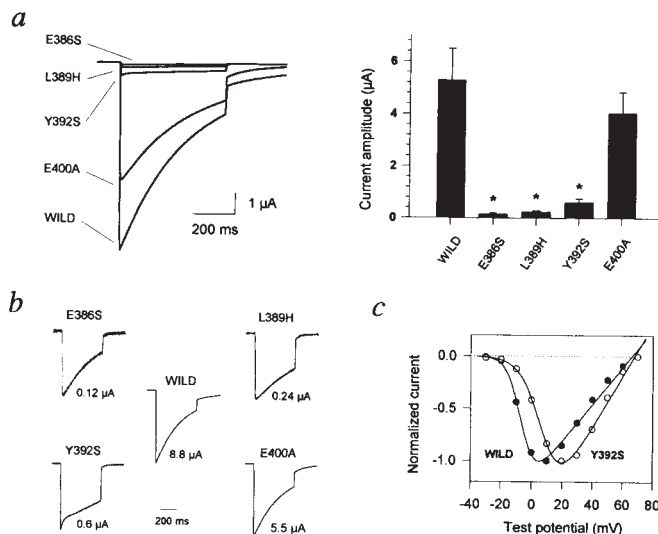


FIG. 4 Mutagenesis of the β -subunit interaction site on the α_{1A} subunit alters the effects of α_1 - β subunit coexpression. **a**, Barium currents of α_{1A} (WILD) or mutated α_{1A} -subunits (E386S, L389H, Y392S and E400A) coexpressed with α_{2B} - and β_{1B} -subunits in *Xenopus* oocytes. Left panel shows superimposed representative traces of Ba^{2+} currents evoked from a holding potential of -90 mV to a +10 mV test potential (TP). Right panel shows average peak currents obtained ($n = 7$ to 9 oocytes). Statistically significant reductions ($P < 0.05$, *t*-test) from wild-type α_{1A} expression levels are denoted by asterisks. Error bars are s.e.m. **b**, Same representative current traces as in **a**, illustrating changes in inactivation kinetics. **c**, Normalized average current-voltage relationship from α_{1A} (WILD) or mutated α_{1A} -subunit (Y392S) to show shift in peak currents induced by the Y392S point mutation. Smooth curves were generated assuming an activation curve of a Boltzmann type $I_{\text{Ba}} = [g(\text{TP} - E)]/[1 + \exp(-(\text{TP} - V_{1/2})/k)]$ with g , the maximum normalized conductance ($g_{\text{WILD}} = 0.018$ and $g_{\text{Y392S}} = 0.024$), E , the reversal potential ($E_{\text{WILD}} = 66$ mV and $E_{\text{Y392S}} = 68$ mV), and $V_{1/2}$, the potential of half-activation ($V_{1/2, \text{WILD}} = -6.4$ mV and $V_{1/2, \text{Y392S}} = 7.6$ mV) and k , the range of potential for an *e*-fold change around $V_{1/2}$ ($k_{\text{WILD}} = 4.6$ mV and $k_{\text{Y392S}} = 6.7$ mV).

METHODS. To mutate the full-length α_{1A} -subunit, pSPCBI-2 (ref. 12), base pairs 1,282–1,745 were amplified by PCR and subcloned into a T-vector made from Bluescript SK⁺. Mutagenic primers and a selection primer, 5'-CATGGTCAATCATGCTTATCGATAC-3', which eliminates the *HindIII* site on the recombinant molecule, were used. The 1,416–1,723 *BsmI* fragment encoding the motif in α_{1A} was then replaced with mutated *BsmI* fragments from the T-vector constructs. To facilitate cloning, the third *BsmI* site in the 3' non-coding region was deleted by digestion at two flanking *BamHI* sites, followed by recircularization of the plasmid. This *BamHI* fragment was restored after subcloning the mutated *BsmI* fragments. The mutated regions were sequenced in both directions on an Applied Biosystems Inc. Automated Sequencer. Complementary RNAs were transcribed *in vitro* using SP6 RNA polymerase with the rabbit brain α_{1A} plasmid or α_{1A} mutants E386S, L389H, Y392S and E400A, and T7 RNA polymerase with rat brain α_{2B} and β_{1B} plasmids. Wild-type or mutant α_{1A} -subunits were co-injected with α_{2B} and β_{1B} into stage V or VI *Xenopus* oocytes at concentrations: 0.6 $\mu\text{g ml}^{-1}$ α_{1A} or mutants, 0.4 $\mu\text{g ml}^{-1}$ α_{2B} and 0.2 $\mu\text{g ml}^{-1}$ β_{1B} ; about 50 nl was injected per cell. Ba^{2+} currents were recorded by a standard two-microelectrode voltage-clamp using a Dagan amplifier (TEV-200). Voltage and current electrodes (0.5–2 M Ω tip resistance) were filled with 3 M KCl. The bath solution was (in mM): $\text{Ba}(\text{OH})_2$, 40; NaOH, 50; KCl, 2; niflumic acid, 1; EGTA, 0.1; HEPES, 5; pH 7.4, adjusted with methanesulphonic acid. Records were filtered at 0.2 to 0.5 kHz and sampled at 1–2 kHz. Leak and capacitance currents were subtracted off-line by a P/4 protocol. Ba^{2+} current through endogenous channels was less than 10 nA (no injection) and average Ba^{2+} current was 40 nA upon injection of β_{1B} and α_{2B} cRNAs.

the β -subunit was determined by the overlay assay described above. The β -subunit interaction was reduced with the E386S_{FP}, L389H_{FP} and Y392S_{FP} mutants compared with the wild-type epitope (Fig. 3a, b). This reduction was greatest with the Y392S_{FP} mutation, which required prolonged exposure of the film for visualization. No reduction in intensity was observed with E400A_{FP}. Densitometric scans of autoradiogram band intensities normalized for protein concentration and averaged over three experiments measured a 27-fold reduction in β -subunit binding to Y392S_{FP} compared with the wild-type epitope. Binding to mutants E386S_{FP} and L389H_{FP} were both reduced 1.7-fold, whereas a 1.3-fold enhancement was measured with E400A_{FP}.

Coexpression of α_{1A} with a β -subunit dramatically increases the functional expression¹² and alters the voltage-dependent and kinetic properties of the channel in *Xenopus* oocytes (not shown). Alteration of the β -subunit binding motif on α_{1A} should therefore alter the modulatory contribution by the β -subunit. A deletion of 30 amino acids that includes the interaction motif (α_{1A} 377–406) or a smaller 16-amino-acid deletion (α_{1A} 377–393) of part of the motif had the result that no detectable current (<10 nA) was observed on coexpression with the β_{1b} -subunit in *Xenopus* oocytes (not shown). To determine more accurately the functional importance of the motif, the four point mutations described above were introduced into the full-length α_{1A} -subunit. Coexpression of E386S _{α_{1A}} , L389H _{α_{1A}} or Y392S _{α_{1A}} with α_{2b} - and β_{1b} -subunits resulted in significantly reduced peak currents compared with the wild-type α_{1A} -subunit (Fig. 4a). Average reductions in current expression were: E386S _{α_{1A}} , 36-fold; L389H _{α_{1A}} , 22-fold; Y392S _{α_{1A}} , 9-fold; E400A _{α_{1A}} , 1.3-fold. Although autoradiogram band intensities of β -subunit overlay were also reduced for mutations E386S, L389H and Y392S, no quantitative correlations were attempted because of the vastly different conditions of these assays. Mutant Y392S _{α_{1A}} altered the inactivation kinetics of the channel (Fig. 4b). At test pulses to +10 mV, Ba²⁺ current inactivated with similar average time constants of (mean $\pm$ s.d.) τ = 350 $\pm$ 59 ms (wild-type α_{1A} , n = 7), τ = 290 $\pm$ 69 ms (E386S _{α_{1A}} , n = 9), τ = 434 $\pm$ 158 ms (L389H _{α_{1A}} , n = 8) and τ = 522 $\pm$ 119 ms (E400A _{α_{1A}} , n = 7). However, Ba²⁺ current conducted by Y392S _{α_{1A}} consistently inactivated along two components, τ_1 = 22 $\pm$ 4 ms (15% of the total current) and τ_2 = 1,806 $\pm$ 682 ms (85% of the total current). In addition to this change in inactivation kinetics, this Y392S mutation also shifted the voltage-dependence of activation (Fig. 4c). All currents, independent of magnitude, peaked at a test potential of +10 mV, except for Y392S _{α_{1A}} , which peaked at +20 mV. More precise estimates of Y392S _{α_{1A}} peak currents were obtained by a fit of the data and showed a general shift by 15 mV towards depolarized test potentials. Although the Y392S mutation greatly modified the biophysical properties of $\alpha_{1A}\alpha_{2b}\beta_{1b}$ complexes, this was not due to a complete loss of α_1 - β -subunit interaction. β_{1b} -subunit still overlaid on the mutant epitope (Fig. 3b) and also, peak current amplitudes with Y392S _{α_{1A}} α_{2b} were enhanced from (mean $\pm$ s.e.m.) -10 ± 4 nA (n = 4) to -613 ± 137 nA (n = 8) by β_{1b} -subunit coexpression. Ba²⁺ currents obtained in the absence of the β_{1b} -subunit were, however, too small for accurate description of the current properties.

The non-conserved residues interspersed among conserved amino acids of the β -subunit interaction motif may be important for the variation in effects of β -subunits on different α_1 -subunits. For example, when the α_{1E} -subunit is coexpressed with the β -subunit, shifts in voltage-dependence of activation and inactivation occur, but no appreciable stimulation of current expression is observed⁹. Sequence variations of the motif in the carp skeletal muscle α_1 -subunit²¹ in position 4 (E $\rightarrow$ D) and in an α_1 subunit (doc-4)²² of torpedo electric organ at positions 7 (L $\rightarrow$ F) and 9 (G $\rightarrow$ R) may indicate differences in modulation by β -subunits in these non-mammalian channels or a structural divergence in their respective β -subunits to accommodate these substitutions. Our results show that substitutions of critical residues in the

β -subunit interaction motif on the Ca²⁺ channel α_1 -subunit alter the ability of the α_1 -subunit to be modulated by the β -subunit. The properties altered by these mutations correlate well with those modified by β -subunits on coexpression with α_1 -subunits^{4–12}. These findings suggest a critical role for the conserved interaction site on the I–II cytoplasmic linker of the α_1 -subunit in voltage-sensitive calcium-channel modulation by the β -subunit. □

Received 1 November; accepted 21 December 1993.

1. Catterall, W. A., Seagar, M. J. & Takahashi, M. *J. biol. Chem.* **263**, 3535–3538 (1987).
2. Campbell, K. P., Leung, A. T. & Sharp, A. H. *Trends Neurosci.* **11**, 425–430 (1988).
3. Witcher, D. R. *et al. Science* **261**, 486–489 (1993).
4. Lacerda, A. E. *et al. Nature* **352**, 527–530 (1991).
5. Perez-Reyes, E. *et al. J. biol. Chem.* **267**, 1792–1797 (1992).
6. Williams, M. E. *et al. Neuron* **8**, 71–84 (1992).
7. Castellano, A., Wei, X., Birnbaumer, L. & Perez-Reyes, E. *J. biol. Chem.* **268**, 3450–3455 (1993).
8. Castellano, A., Wei, X., Birnbaumer, L. & Perez-Reyes, E. *J. biol. Chem.* **268**, 12359–12366 (1993).
9. Soong, T.-W. *et al. Science* **260**, 1133–1136 (1993).
10. Stea, A. *et al. Neuropharmacology* **32**, 1103–1116 (1993).
11. Nishimura, S., Takeshima, H., Hofmann, F., Flockerzi, V. & Imoto, K. *FEBS Lett.* **324**, 283–286 (1993).
12. Mori, Y. *et al. Nature* **350**, 398–402 (1991).
13. Neely, A., Wei, X., Olcese, R., Birnbaumer, L. & Stefani, E. *Science* **262**, 575–578 (1993).
14. Pragnell, M., Sakamoto, J., Jay, S. D. & Campbell, K. P. *FEBS Lett.* **291**, 253–258 (1991).
15. Leung, A. T., Imagawa, T. & Campbell, K. P. *J. biol. Chem.* **262**, 7943–7946 (1987).
16. Ellis, S. B. *et al. Science* **241**, 1661–1664 (1988).
17. Dubel, S. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **89**, 5058–5062 (1992).
18. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. *molec. Biol.* **215**, 403–410 (1990).
19. Biel, M. *et al. FEBS Lett.* **269**, 409–412 (1990).
20. Hui, A. *et al. Neuron* **7**, 35–44 (1991).
21. Grabner, M. *et al. Proc. natn. Acad. Sci. U.S.A.* **88**, 727–731 (1991).
22. Horne, W. A. *et al. Proc. natn. Acad. Sci. U.S.A.* **90**, 3787–3791 (1993).
23. Marchuk, D., Drumm, M., Saulino, A. & Collins, S. F. *Nucleic Acids Res.* **19**, 1154 (1991).

ACKNOWLEDGEMENTS. We thank L. Birnbaumer and X. Wei for the α_{1C} -subunit cDNA; S. D. Kahl and W. Guo for technical assistance; and B. A. Adams, T. Hoshi, S. L. Roberds, E. Shibata, A. Stea and D. R. Witcher for comments on the manuscript. K.P.C. and T.T. are investigators of HHMI; T.P.S. is an HHMI International Research Scholar and is also supported by a grant from the MRC of Canada.

A 13-amino-acid motif in the cytoplasmic domain of Fc γ RIIB modulates B-cell receptor signalling

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THE Fc receptor on B lymphocytes, Fc γ RIIB (β 1 isoform), helps to modulate B-cell activation triggered by the surface immunoglobulin complex^{1,2}. Crosslinking of membrane immunoglobulin by antigen or anti-Ig F(ab')₂ antibody induces a transient increase in cytosolic free Ca²⁺, a rise in inositol-3-phosphate, activation of protein kinase C, and enhanced protein tyrosine phosphorylation^{3–5}. Crosslinking Fc γ RIIB with the surface immunoglobulin complex confers a dominant signal that prevents or aborts lymphocyte activation triggered through the ARH-1 motifs of the signal transduction subunits Ig- α and Ig- β . Here we show that Fc γ RIIB modulates membrane immunoglobulin-induced Ca²⁺ mobilization by inhibiting Ca²⁺ influx, without changing the pattern of tyrosine phosphorylation. A 13-amino-acid motif in the cytoplasmic domain of Fc γ RIIB is both necessary and sufficient for this effect. Tyrosine

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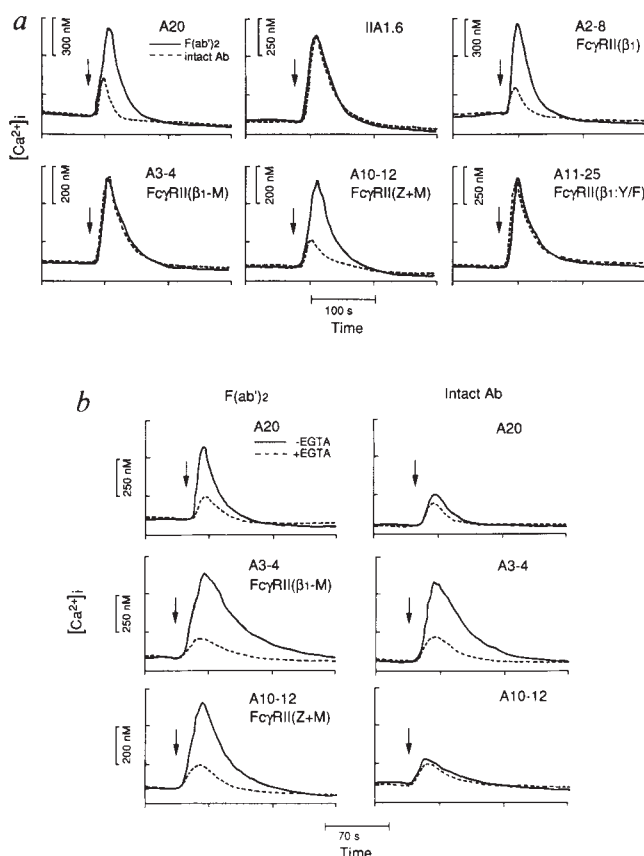


FIG. 1 Calcium mobilization after crosslinking of mIg or co-crosslinking with FcγRII. Ca²⁺ mobilization of non-transfected and transfected cells stimulated by whole and F(ab')₂ anti-mIg antibodies (a), and the effect of EGTA on the Ca²⁺ mobilization induced by these antibodies (b). METHODS. Cells (1 × 10⁶ per ml) were loaded with 3 μM Fura-2 and stimulated with rabbit intact (40 μg ml⁻¹) and F(ab')₂ (25 μg ml⁻¹) anti-mIgG antibodies. Intracellular Ca²⁺ levels were recorded with fluorescence spectrophotometer (Hitachi F2000). In the presence of the anti-FcγRIIB mAb, 2.4G2 (4 μg ml⁻¹), Ca²⁺ mobilization patterns induced by intact antibodies were essentially the same as those induced by F(ab')₂. For chelation of extracellular Ca²⁺, EGTA (1 mM) was added 1 min before ligand stimulation. Crosslinking FcγRIIB by 2.4G2 evoked no detectable intracellular signalling (data not shown).

at residue 309 in this motif is phosphorylated upon co-crosslinking with surface immunoglobulin; mutation of this residue aborts the inhibitory effect of FcγRIIB. This inhibition is directly coupled to signalling mediated through Ig-α and Ig-β as evidenced by chimaeric IgM/α and IgM/β molecules. The 13-residue motif in FcγRIIB controls lymphocyte activation by inhibiting a Ca²⁺ signalling pathway triggered through ARH-1 motifs as a result of recruitment of novel SH2-containing proteins that interact with this FcγRIIB cytoplasmic motif.

Anti-mIg (whole IgG directed towards membrane immunoglobulin) which crosslinks surface FcγRII with mIg, inhibits Ca²⁺ mobilization in the A20 B-lymphoma cell line (Fig. 1a). This inhibition is reversed by the anti-FcγRII monoclonal antibody 2.4G2, which prevents binding of the intact Fc domain of anti-mIg to FcγRII (ref. 6, and data not shown). Stimulation of mIg evokes Ca²⁺ release from intracellular stores and Ca²⁺ influx from the outside⁷. We stimulated A20 cells in the presence and absence of EGTA to distinguish between these Ca²⁺ sources. Incubation with EGTA decreased Ca²⁺ mobilization fourfold upon crosslinking of mIg with anti-mIg F(ab')₂, whereas even in the presence of EGTA, Ca²⁺ mobilization induced by adding whole anti-mIg was roughly the same (Fig. 1b). This indicates that the effect of FcγRII is primarily to inhibit Ca²⁺ influx across the plasma membrane. In contrast, tyrosine-phosphorylated pro-

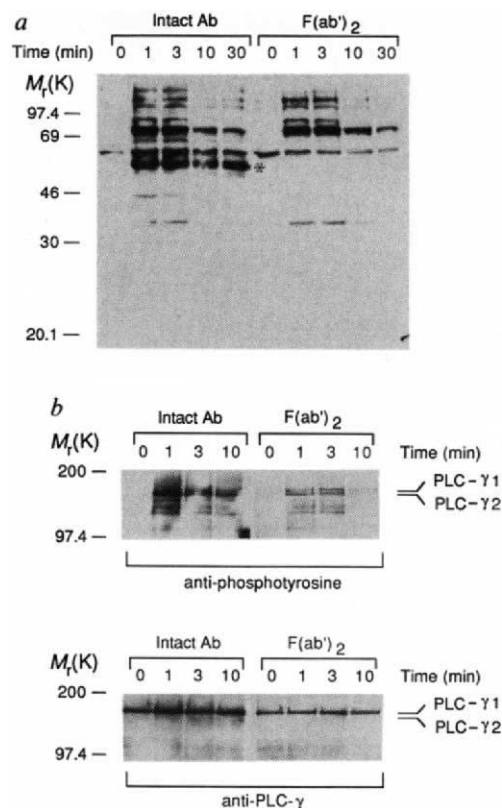


FIG. 2 Tyrosine phosphorylation after cross-linking of mIg or co-crosslinking with FcγRII. Immunoblot analysis of whole-cell lysates (a) or phospholipase Cγ (b) with antiphosphotyrosine antibody.

METHODS. A20 cells (5.0 × 10⁶ per ml) were stimulated by intact (50 μg ml⁻¹) or F(ab')₂ (25 μg ml⁻¹) anti-mIg antibodies at 37 °C. 10-μl aliquots were lysed with 2 × Laemmli's sample buffer at the indicated time. Proteins were resolved on 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with a monoclonal anti-phosphotyrosine antibody 4G10 (UBI) and anti-mouse antibody-HRP conjugate. Phosphotyrosine-containing proteins were visualized with an ECL system (Amersham). The band in a indicated by an asterisk is the heavy chain of the IgG molecule used for stimulation. For the phospholipase Cγ (PLC-γ) analysis (b), A20 cells (1.6 × 10⁷ per ml) were stimulated by whole (40 μg ml⁻¹) or F(ab')₂ (20 μg ml⁻¹) antibody at 37 °C. 500-μl aliquots were lysed in 1% NP-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM Na₃VO₄, 5 mM NaF. Insoluble material was removed by centrifugation and phospholipase Cγ was immunoprecipitated with anti-phospholipase Cγ antiserum (UBI) and protein A-Sepharose. Immunoprecipitates were resolved on 7.5% SDS-PAGE, blotted and incubated with 4G10 (top). The filter was stripped and incubated with the anti-phospholipase Cγ antiserum followed by anti-rabbit antibody-HRP conjugate (bottom). The anti-phospholipase Cγ antiserum used here reacts more strongly with the phospholipase Cγ1 isoform than with γ2, which is the major isoform in B cells.

teins from A20 cell lysates stimulated by whole or F(ab')₂ anti-mIg antibody showed no significant change. There was no difference in the tyrosine phosphorylation of phospholipases C-γ1 or -γ2, enzymes involved in intracellular Ca²⁺ release^{8,9}, when A20 cells were stimulated by whole or F(ab')₂ antibodies (Fig. 2). This supports our earlier conclusion that FcγRIIB regulates Ca²⁺ flux into the cell.

To define the region(s) in FcγRII responsible for this inhibition, complementary DNA encoding a 13-residue internal deletion of the FcγRII cytoplasmic domain was transfected into an FcγRII-negative mutant of the A20 B-cell lymphoma IIA1.6 (ref. 10) to obtain A3-4 (Fig. 3). In contrast to wild-type FcγRII, this internal deletion mutant had no effect on Ca²⁺ influx induced by crosslinking FcγRII with mIg (compare panels A3-4 with A20 and A2-8 in Fig. 1). To determine whether this 13-amino-acid sequence was sufficient to inhibit Ca²⁺ mobilization,

we constructed a chimaeric receptor in which the first 18 residues and the following 13 residues of the cytoplasmic domain were derived from the ζ -chain of the T-cell antigen receptor (TCR)/CD3 complex and from Fc γ RII, respectively (Fig. 3a). Stable cell lines were obtained after transfection into an Fc γ RII-negative A20 B-cell line (A10-12). This chimaeric receptor inhibited

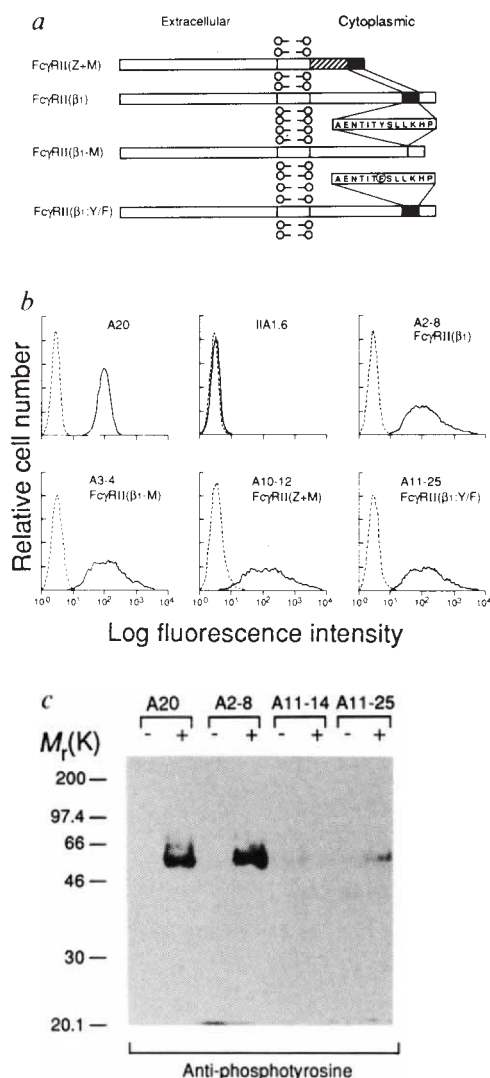


FIG. 3 Expression and tyrosine phosphorylation of Fc γ RIIB constructs. **a**, Schematic representation of Fc γ RII cDNA constructs; **b**, FACS analysis of IIA1.6 (Fc γ RIIB) cells transfected with wild-type and mutant Fc γ RIIB cDNAs; and **c**, tyrosine phosphorylation of wild-type and mutant Fc γ RIIB. **METHODS.** Mutant murine Fc γ RII cDNAs shown in **a** were constructed using polymerase chain reaction (PCR) methods. All constructs were confirmed by sequencing. Fc γ RII (Z+M), and Fc γ RII (β 1-M), and Fc γ RII (β 1:Y/F) contain the extracellular and transmembrane domains of Fc γ RII (β 1). The cytoplasmic domain of Fc γ RII (β 1-M) has the internal deletion of 13 residues (amino acids 303–315 in the β 1 isoform of Fc γ RII); that of Fc γ RII (Z+M) is composed of the first 18 and the following 13 residues from the cytoplasmic domain of human ζ chain of TCR/CD3²⁴ (amino acids 53–68 in human ζ and two additional serines, shown in hatched region) and Fc γ RII (ref. 25) (AENTITYSLLKHP), respectively. These cDNAs were cloned together with the neomycin-resistant gene into pcEXV-3. Fc γ RII (β 1:Y/F) bears a point mutation (309 Tyr $\rightarrow$ Phe) in the cytoplasmic domain. IIA1.6 cells were transfected by electroporation and neomycin-resistant clones were characterized by FACS analysis using the monoclonal antibody (mAb) 2.4G2 A20 cells and IIA1.6 transformants expressing a wild-type (A2-8) and a Y309F mutant (A11-14 and A11-25) were stimulated with 10 μ g ml⁻¹ anti-IgG intact antibody for 3 min. Fc γ RIIB was immunoprecipitated with 2.4G2, resolved on 10% SDS-PAGE, transferred to nitrocellulose and probed with the antiphosphotyrosine antibody RC20 (Transduction Lab, Lexington).

Ca²⁺ mobilization induced by crosslinking Fc γ RII with mIgG by blocking Ca²⁺ influx (Fig. 1), like the wild-type receptor. The 13-residue motif in the cytoplasmic domain of Fc γ RII must therefore be sufficient to inhibit mIg-induced Ca²⁺ mobilization.

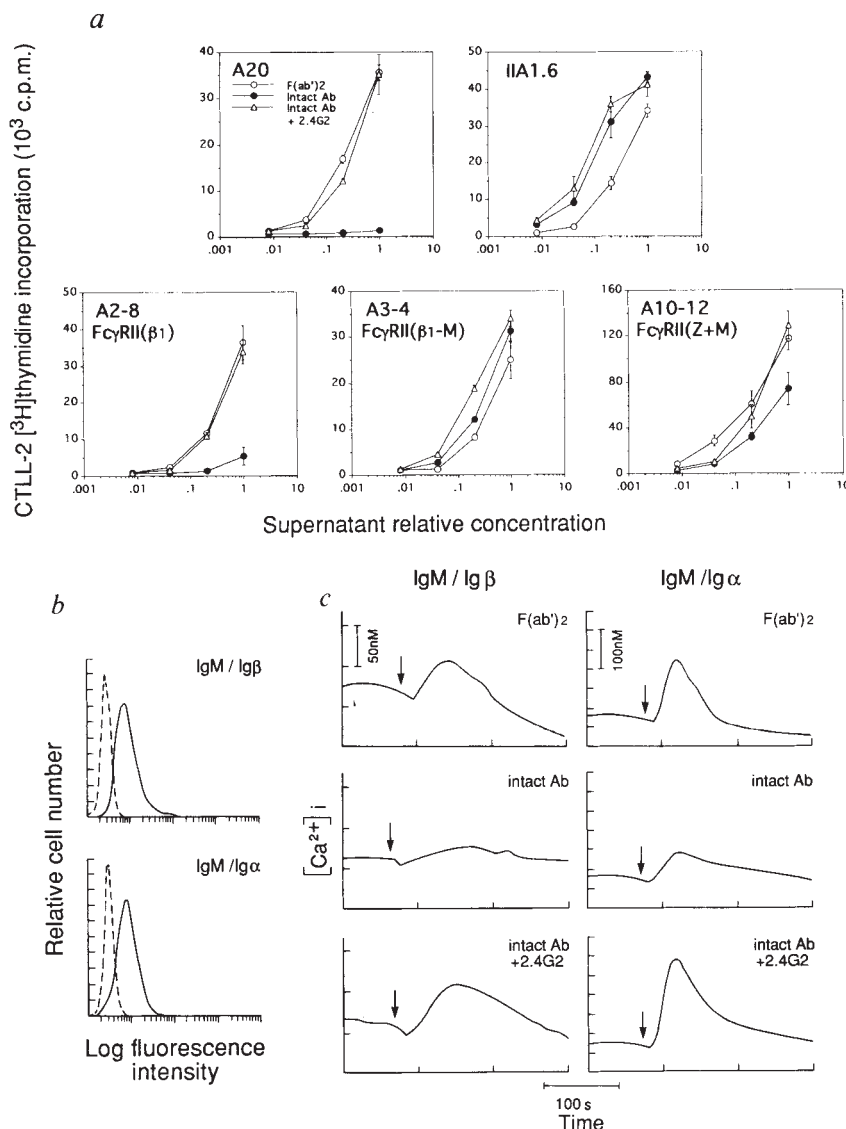
The surface immunoglobulin complex is composed of membrane immunoglobulin and the associated signal transduction subunits Ig- α (mb1) and Ig- β (B29) (ref. 3), which become tyrosine-phosphorylated upon stimulation by mIg¹¹. Similarly, crosslinking of mIg with Fc γ RIIB results in tyrosine phosphorylation of this Fc receptor (Fig. 3c). To investigate whether Tyr 309 in this 13-residue motif could be involved in the regulation of mIg-mediated Ca²⁺ mobilization, we created a Fc γ RII construct bearing a point mutation (Tyr 309 $\rightarrow$ Phe) (Fig. 3a). This receptor was transfected into the A20 Fc γ RIIB-negative cell line IIA1.6 to obtain A11-25 and A11-14. Even though cell-surface expression of the Fc γ RII (β 1:Y/F) mutant was comparable to that of parental A20 or A2-8 (Fig. 3b), these mutants had significantly reduced tyrosine phosphorylation of Fc γ RIIB (Fig. 3c) and did not affect mIg-mediated Ca²⁺ mobilization (Fig. 1).

The inhibition of B-cell activation resulting from crosslinking Fc γ RII with mIg involves both early and late signalling responses^{12,13}. To assess the contribution of Fc γ RIIB inhibition on late responses, we analysed Fc γ RIIB mutant A20 lines for effect on secretion of interleukin-2 (IL-2) stimulated by crosslinking mIg. Wild-type Fc γ RII modulated IL-2 secretion in A20 cells and IIA1.6 cells transfected with wild-type IIB (Fig. 4a). By contrast, deletion of the 13-amino-acid motif essential for Ca²⁺ regulation also destroyed the modulation of IL-2 secretion. The chimaeric receptor Fc γ RII(Z+M) was able to regulate mIg-induced IL-2 secretion, but it was only 50% as effective as the wild-type receptor (Fig. 4a). These results indicate that whereas the 13-residue segment in the cytoplasmic domain of Fc γ RII is required for regulation of late responses, other domains of the receptor are necessary to maximize these responses.

The ARH-1 motif in the cytoplasmic domains of mIg-associated Ig- α and Ig- β chains¹⁴ carries sufficient structural information to activate signalling pathways^{15–19}. The cytoplasmic domains of Ig- α and Ig- β interact with different cytoplasmic effector proteins to give different responses^{19,20}. To establish whether Fc γ RII can influence Ig- α - and Ig- β -dependent signalling, we constructed chimaeric IgM/Ig- α and IgM/Ig- β receptors in which the extracellular and transmembrane domains were derived from mIgM and the cytoplasmic domain from either Ig- α or Ig- β . These molecules were expressed in the A20 B-lymphoma cell line (Fig. 4b). To avoid association of the chimaeric receptor molecules with endogenous Ig- α and Ig- β , we introduced the mutations Tyr-Ser to Val-Val in the transmembrane domain of mIgM (ref. 19). Crosslinking of these chimaeric molecules with anti-IgM F(ab')₂ provoked Ca²⁺ mobilization, but crosslinking Fc γ RII with IgM/Ig- α and IgM/Ig- β using intact antibody inhibited this mobilization, an effect that could be reversed in the presence of an anti-Fc γ RII antibody (Fig. 4c). A20 B cells transfected with wild-type IgM had Ca²⁺ mobilized as a result of stimulation by anti-IgM F(ab')₂. This mobilization was modulated when Fc γ RII was crosslinked with IgM. IgM/Ig- β -Y/F, in which a tyrosine at position 206 is changed to phenylalanine, did not evoke Ca²⁺ mobilization (data not shown). These results indicate that Fc γ RII prevents mIgM-induced Ca²⁺ activation by interfering with the signal initiated through the ARH-1 motif in the cytoplasmic domains of Ig- α and Ig- β .

Our results show that the active site responsible for Fc γ RII inhibition of mIg-induced Ca²⁺ mobilization is a linear 13-amino-acid sequence. Under physiological conditions, this Ca²⁺ channel is regulated through phosphorylation by cyclic AMP-dependent protein kinase²¹. There was no detectable change in cAMP levels in A20 cells after stimulation of whole or F(ab')₂ anti-mIg antibodies (data not shown), suggesting that this 13-

FIG. 4 a, IL-2 secretion after crosslinking of mlg or co-crosslinking with Fc γ RII. IL-2 secretion of non-transfected and transfected cells by whole and F(ab')₂ anti-mlgG antibodies. b and c, Modulation of calcium mobilization through chimaeric molecules IgM/Ig- α and IgM/Ig- β by Fc γ RII. Cell-surface expression of transfected IgM/Ig- α and IgM/Ig- β on A20 cells (b) and Ca²⁺ mobilization stimulated by whole and F(ab')₂ anti-hlgM antibodies (c). METHODS. a, Cells (5 $\times$ 10⁵ per ml) were stimulated by the indicated antibodies (10 μ g ml⁻¹ intact, 5 μ g ml⁻¹ F(ab')₂, and 10 μ g ml⁻¹ 2.4G2 antibodies) for 18 h at 37 °C. IL-2 activity in serial dilutions of the culture supernatant was measured by [³H]thymidine incorporation using the IL-2-dependent cell line CTLL-2, as described²⁶. Experiments were done three times for each cell line, and the mean and s.e.m. of triplicate points are shown. Equivalent amounts of surface IIB expression were seen for all cases (Fig. 3b). b and c, Chimaeric IgM/Ig- α cDNA is composed of human κ - and μ -chimaeric chains against phosphorylcholine. The extracellular, transmembrane and cytoplasmic domains of the chimeric μ chain are derived from wild-type μ chain, mutated transmembrane μ chain (replacement of both tyrosine 587 and serine 588 with valine) and murine cytoplasmic Ig- α (amino-acids 160–220), respectively. IgM/Ig- β is the same as IgM/Ig- α , except that the cytoplasmic domain is composed of amino-acids 181–228 murine Ig- β . DNAs were transfected into A20 cells by electroporation, and resistant clones were characterized by FACS analysis using rabbit anti-human (h)IgM antibody. Ca²⁺ mobilization was determined as described in the legend to Fig. 1 (ref. 19). Rabbit whole (80 μ g ml⁻¹) and F(ab')₂ (50 μ g ml⁻¹) anti-hlgM antibodies were used for stimulation (middle and top panels). Cells were preincubated with 2.4G2 (5 μ g ml⁻¹) for 5 min before application of intact antibody (bottom panel).



residue motif regulates Ca²⁺ influx through a cAMP-independent pathway. Tyrosine 309 is likely to be important for inhibition by Fc γ RIIB. The amino-acid sequence YSLI that includes this tyrosine corresponds to one of the consensus sequences that can bind proteins with an SH2 domain²², so phosphorylation of Tyr 309 may be critical for recruiting SH2-containing proteins to initiate control without affecting the overall pattern of tyrosine phosphorylation. Fc γ RIIB probably acts by recruiting novel SH2-containing proteins and not by competing for SH2-containing proteins that interact with Ig- α and Ig- β . Identification of these proteins will elucidate an important inhibitory pathway in

B-cell activation.

The Fc γ RII-dependent pathway of B-cell activation is important in lymphocyte regulation during the antibody-secretion phase of a humoral immune response. Feedback inhibition of B-cell proliferation and antibody secretion by cognate antibody may help prevent autoimmune antibody production; further, the 13-amino-acid inhibitory motif is the only sequence that is conserved between the murine and human Fc γ RII cytoplasmic domains²³. Understanding the interaction of this motif with cellular components should elucidate the mechanism of inhibition. □

Received 21 October; accepted 17 December 1993.

1. Ravetch, J. V. & Kinet, J.-P. *Rev. Immun.* **9**, 457–492 (1991).
2. Mellman, I. *Curr. Opin. Immun.* **1**, 16–25 (1988).
3. Reth, M. A. *Rev. Immun.* **10**, 97–121 (1992).
4. Desiderio, S. V. *Curr. Opin. Immun.* **4**, 252–256 (1992).
5. Cambier, J. C. & Ransom, J. T. A. *Rev. Immun.* **5**, 175–199 (1987).
6. Wilson, H. A. et al. *J. Immun.* **238**, 1712–1718 (1987).
7. Ransom, J. T. et al. *J. Immun.* **140**, 3150–3155 (1988).
8. Carter, R. H., Park, D. J., Rhee, S. G. & Fearon, D. T. *Proc. natn. Acad. Sci. U.S.A.* **88**, 2745–2749 (1991).
9. Ransom, J. T., Harris, L. K. & Cambier, J. C. *J. Immun.* **137**, 708–714 (1986).
10. Jones, B., Tite, J. P. & Janeway, C. A. *J. Immun.* **136**, 348–356 (1986).
11. Gold, M. R., Matsuuchi, L., Kelly, R. B. & DeFranco, A. L. *Proc. natn. Acad. Sci. U.S.A.* **88**, 3436–3440 (1991).
12. Hunziker, W., Koch, T., Whitney, J. A. & Mellman, I. *Nature* **345**, 628–632 (1990).
13. Amigorena, S. et al. *Science* **256**, 1808–1812 (1992).
14. Reth, M. *Nature* **338**, 383–384 (1989).
15. Letourneur, F. & Klausner, R. D. *Science* **255**, 79–82 (1992).

16. Romeo, C., Amiot, M. & Seed, B. *Cell* **68**, 889–897 (1992).
17. Irving, B. A., Chan, A. C. & Weiss, A. *J. exp. Med.* **177**, 1093–1103 (1993).
18. Kolanus, W., Romeo, C. & Seed, B. *EMBO J.* **11**, 4861–4868 (1992).
19. Sanchez, M. et al. *J. exp. Med.* **178**, 1049–1055 (1993).
20. Clark, M. R. et al. *Science* **258**, 123–126 (1992).
21. Kameyama, M., Hoffmann, F. & Trautwein, W. *Pflügers Arch.* **405**, 285–293 (1985).
22. Songyang, Z. et al. *Cell* **72**, 767–778 (1993).
23. Brooks, D. G., Qiu, W. Q., Luster, A. D. & Ravetch, J. V. *J. exp. Med.* **170**, 1369–1386 (1989).
24. Weissman, A. M. et al. *Proc. natn. Acad. Sci. U.S.A.* **85**, 9709–9713 (1988).
25. Ravetch, J. V. et al. *Science* **234**, 718–725 (1986).
26. Lai, M.-Z. et al. *J. Immun.* **139**, 3973–3980 (1987).

ACKNOWLEDGEMENTS. T.M. and T.K. contributed equally to this work. We thank C. A. Janeway for A20 B lymphoma and IIA1.6 cell lines, and M. Kurosaki for technical assistance. These studies were supported by grants to J.V.R. and M.C.N. from the NIH and to M.C.N. from the Howard Hughes Medical Institute. T.K. is an Adler Fellow and Cohen Fellow in Biomedical Research; T.M. is supported by JSPS postdoctoral fellowships for research abroad.

Transcription factor b (TFIIH) is required during nucleotide-excision repair in yeast

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NUCLEOTIDE-excision repair (NER)¹ is an important cellular defence mechanism against mutagenesis and carcinogenesis. The essential yeast genes *RAD3* (ref. 2) and *SSL2* (*RAD25*)^{3,4}, homologues of the human xeroderma pigmentosum genes *XPB*^{5,6} and *XPB*⁷ respectively, have been implicated in NER in yeast. The products of these genes are also subunits of (Rad3 protein) or associate with (Ssl2 protein) purified yeast RNA polymerase II transcription initiation factor b, the counterpart of human TFIIH⁸. Rad3 and Ssl2 proteins may participate directly in NER. Alternatively, they may function exclusively as transcription factors that support NER by influencing the expression of other NER genes. Here we show that defective NER in *rad3* mutant extracts can be specifically complemented by purified transcription factor b. Similarly, defective NER in *ssl2* mutant extracts is corrected by purified factor b/Ssl2 complex. These results support a direct role of factor b during NER in yeast. Hence, factor b (TFIIH) has a dual role in transcription and NER.

NER can be demonstrated in yeast cell-free extracts by monitoring repair synthesis in plasmid DNA treated with DNA-damaging agents⁹. We used *N*-acetoxy-2-acetylaminofluorene (AAAF) as a source of base damage¹⁰⁻¹³ to evaluate NER in extracts of mutants defective in the *SSL2* and *RAD3* genes of *Saccharomyces cerevisiae*. Extracts of wild-type cells are proficient in the repair of acetylaminofluorene (AAF) adducts (Fig. 1a, lane 5). In contrast, extracts of an ultraviolet radiation-sensitive mutant designated *ssl2-xp* (ref. 3) (Fig. 1a, lane 3) and of two different ultraviolet-sensitive *rad3* mutants² (Fig. 1b, lanes 1 and 5) have a marked deficiency in repair synthesis. The *rad3* extracts were complemented by crude extract from *rad3* cells transformed with a plasmid carrying the wild-type *RAD3* gene (Fig. 1b, lanes 2 and 6). These results confirm earlier studies that established a requirement for the *RAD3* gene during NER². These results also provide direct evidence of a requirement for the *SSL2* gene in NER and are consistent with results showing that *SSL2* is required for the removal of pyrimidine dimers from the genome of yeast cells¹⁴.

The repair-defective phenotype of *rad3* and *ssl2* cells may reflect the direct participation of Rad3 and Ssl2 proteins in NER. However, Rad3 is the 85K (*M*, 85,000) subunit of RNA polymerase II transcription factor b⁸ and a conditional-lethal *rad3* mutant is defective in polymerase II-dependent transcription¹⁵. Like *RAD3*, *SSL2* (*RAD25*) is essential for viability^{3,4} and a conditional-lethal *rad25* mutant is also defective in RNA polymerase II-dependent transcription *in vivo*¹⁶. Finally, Ssl2 protein binds to factor b through a specific interaction with Rad3 protein²⁷. Hence, defective NER in *rad3* and *ssl2* mutants is also consistent with an indirect effect mediated by deficient or defective transcription of other NER genes. To distinguish between these possibilities, we examined complementation of mutant extracts with purified proteins or protein complexes under conditions that preclude coupled transcription-translation of other NER genes in the extracts.

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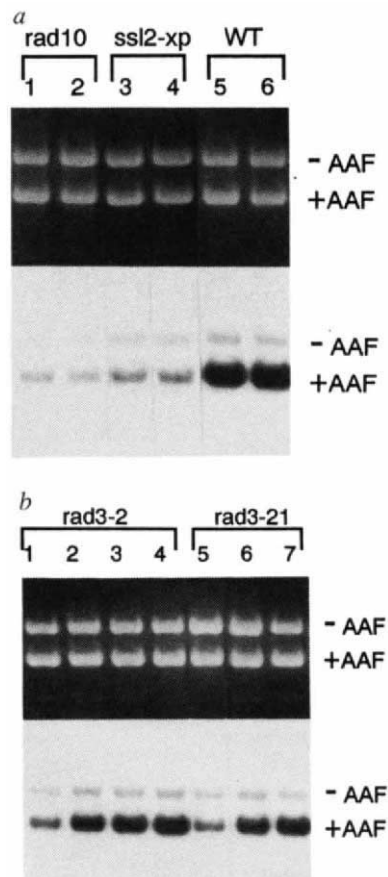


FIG. 1 Complementation of NER in *rad3* mutant extracts. AAAF-damaged DNA was prepared by incubating plasmid pUC18 DNA (100 µg) at 37 °C for 3 h in 1 ml TE buffer⁹ containing 3 µM AAAF. The modified DNA was purified in a 5–10% sucrose gradient as described²². DNA repair synthesis assays were performed with cell-free extracts (250 µg nuclear extract and 50 µg of whole-cell extract containing overexpressed Rad2 protein) as described⁹. a, Repair synthesis in 300 µg yeast extract from *rad10*, *ssl2-xp* or wild-type cells was performed in the absence (lanes 1, 3 and 5) or in the presence of 180 ng (lane 4) or 450 ng (lanes 2 and 6) of factor b. Quantitation of DNA damage-specific repair synthesis⁹ indicates 14–20 fmol dCMP incorporated in mutant extracts and 80–140 fmol dCMP incorporated in wild-type extracts. This range of values was found in all other experiments shown. b, Repair synthesis in 300 µg *rad3* mutant extracts (lanes 1 and 5) was complemented with 50 µg yeast whole-cell extract containing overexpressed Rad3 protein (lanes 2 and 6) or purified factor b (180 ng, lanes 3 and 7; or 450 ng, lane 4). The purified factor b used here consists of 5 major polypeptides of *M*_s 85K, 70K, 55K, 50K and 38K⁸. +AAAF, damaged DNA; -AAAF, undamaged DNA. Top, ethidium bromide-stained gel; bottom, autoradiogram. Yeast strains used were LN203-2 (*rad3-2*)²², S14-2 (*rad3-21*)²³, BJrad10²², KG119 (*ssl2-xp*)³ and CL1265-7C (WT)²⁴.

The cell-free extracts⁹ cannot support transcription or translation as they are depleted of nucleoside triphosphates and amino acids by extensive dialysis. Indeed, we did not detect incorporation of radioactivity from either [α -³²P]UTP or [³⁵S]methionine into RNA or protein respectively, under standard NER conditions (data not shown). Additionally, we monitored repair synthesis of AAAF-treated plasmid DNA in the presence of RNase A, which would preclude coupled transcription-translation *in vitro*. RNase A had no effect on repair synthesis in wild-type (Fig. 2, lanes 1 and 2) or complemented (see later) *rad3* mutant extracts (Fig. 2, lanes 4 and 6).

Purified active Rad3 protein (3 pmol) did not complement defective repair synthesis in extracts of *rad3-21* cells (Fig. 3), but purified factor b (150 fmol) did (Fig. 1b, lanes 3, 4 and 7).

FIG. 2 Effect of RNase

A on NER. DNA repair synthesis was performed in the presence (+) or absence (-) of 1 µg RNase A in wild-type (WT) yeast extracts or *rad3* mutant extracts with (+) or without (-) added transcription factor b. After incubation at 30 °C for 2 h, reaction mixtures were treated with proteinase K and the DNA was purified as described²⁵, except that RNase A treatment after the repair reaction was eliminated. Reaction products were then analysed by 1% agarose gel electrophoresis in the presence of 0.5 µg ml⁻¹ ethidium bromide. DNA in lanes 1, 3 and 4 migrated slightly faster owing to the presence of RNA, which is typically removed by RNase A digestion before electrophoresis. Top, ethidium bromide-stained gel; bottom, autoradiogram.

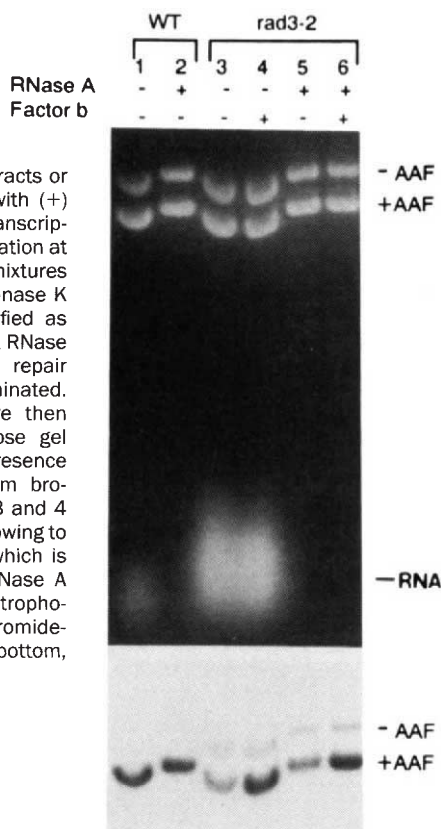
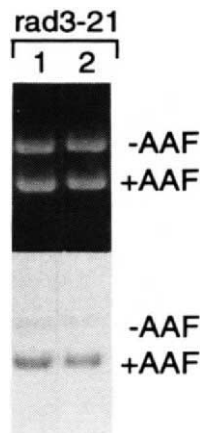


FIG. 3 NER in *rad3* mutant extracts supplemented with purified Rad3 protein. Repair synthesis in 300 µg *rad3-21* mutant extract was done in the absence (lane 1) or presence (lane 2) of 270 ng purified Rad3 protein²⁶. Top, ethidium bromide-stained gel; bottom, autoradiogram.



Complementation was specific to *rad3* extracts, because factor b had no effect on extracts of *rad10* or *ssl2* mutant cells, or wild-type cells (Fig. 1a). Purified Ssl2 protein (200 fmol) did not complement defective NER in *ssl2-xp* mutant extracts (Fig. 4a, lanes 1 and 2). However, 400 fmol of purified factor b/Ssl2 complex resulted in levels of repair synthesis (lane 5) comparable to that in wild-type extracts (Fig. 4b, lane 4). The factor b/Ssl2 complex also corrected defective repair in *rad3* mutant extracts (Fig. 4b, lane 3). Partial correction in *ssl2-xp* extracts was observed with excess purified Ssl2 protein (Fig. 4a, lanes 3 and 4). But excess Ssl2 protein had no effect on *rad3* mutant extracts (Fig. 4b, lane 2).

These results demonstrate that Rad3 and Ssl2 proteins participate directly in NER. Hence, both yeast proteins and, by implication, the human XPD and XPB homologues have dual roles in transcription and NER. The role(s) of Rad3 and Ssl2 proteins in NER are apparently subserved only when these proteins are assembled in or associated with factor b. Endogenous factor b in the *rad3* mutant extracts is highly stable, because a vast excess

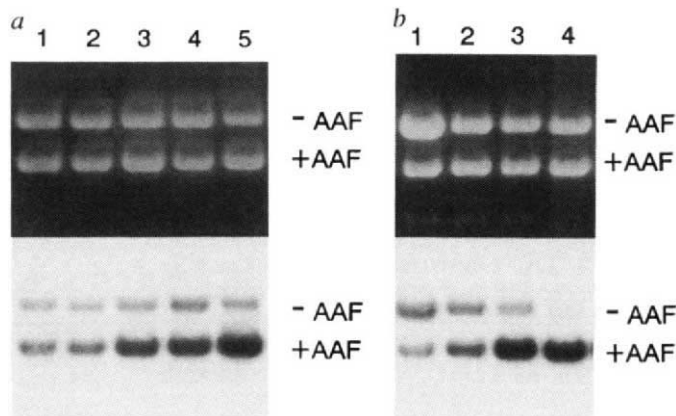


FIG. 4 Complementation of NER in *ssl2* mutant extracts. a, Repair synthesis in 300 µg *ssl2-xp* mutant extract (lane 1) was complemented with 0.2 pmol (lane 2), 0.6 pmol (lane 3) or 1.2 pmol (lane 4) purified Ssl2 protein, or 0.4 pmol purified factor b containing associated Ssl2 protein (lane 5). b, Repair synthesis in 300 µg *rad3-1* mutant extract was done in the absence (lane 1) or presence of 1.2 pmol purified Ssl2 (lane 2) or 1.2 pmol purified factor b containing associated Ssl2 protein (lane 3). Lane 4, repair synthesis in wild-type yeast extract. The purification of Ssl2 protein and factor b complexed with Ssl2 protein will be presented elsewhere (J.Q.S. et al., unpublished data). Top, ethidium bromide-stained gel; bottom, autoradiogram.

of Rad3 protein was unable to substitute for the defective form. Ssl2 protein can exert a limited mass action effect, resulting in partial correction of defective NER in *ssl2-xp* extracts. Thus, Rad3 is apparently more tightly bound than Ssl2 in factor b, consistent with the observation that factor b can be purified with or without Ssl2 protein (J.Q.S. et al., unpublished results).

The essential *Tfbl* and *Ssl1* genes encode the 75K and 50K subunits of factor b respectively^{8,17,18}. Our observation that factor b directly participates in NER predicts that *Tfbl* and *Ssl1* are also likely to be NER proteins. Indeed, viable ultraviolet-sensitive alleles of these genes have been isolated¹⁸ (S. Buratowski, personal communication).

The intimate association between NER and transcription through factor b provides a reasonable biochemical basis for the preferential repair of transcriptionally active genes^{19,21}. However, viable *rad3* mutants show a total defect in pyrimidine-dimer excision². We therefore suggest that factor b may comprise a core 'repairoosome' which participates in transcriptionally coupled and transcriptionally independent NER. A requirement for factor b in both transcription initiation and NER in bulk DNA provides a possible mechanism for limiting transcription when cells have sustained significant DNA damage. □

Received 13 December 1993; accepted 26 January 1994.

1. Friedberg, E. C. *DNA Repair* (Freeman, New York, 1985).
2. Friedberg, E. C., Siede, W. & Cooper, A. J. in *The Molecular and Cellular Biology of the Yeast Saccharomyces: Genome Dynamics, Protein Synthesis, and Energetics*, vol. 1 (eds Broach, J. R., Jones, E. & Pringle, J.), 147-192 (Cold Spring Harbor Laboratory Press, Plainview, New York, 1991).
3. Gulyas, K. D. & Donahue, T. F. *Cell* **69**, 1031-1042 (1992).
4. Park, E. et al. *Proc. natn. Acad. Sci. U.S.A.* **89**, 11416-11420 (1992).
5. Weeda, G. et al. *Cell* **62**, 777-791 (1990).
6. Flejter, W. L., McDaniel, L. D., Johns, D., Friedberg, E. C. & Schultz, R. A. *Proc. natn. Acad. Sci. U.S.A.* **89**, 261-265 (1992).
7. van Duin, M. et al. *Cell* **44**, 913-923 (1986).
8. Feaver, W. J. et al. *Cell* **75**, 1379-1387 (1993).
9. Wang, Z., Wu, X. & Friedberg, E. C. *Proc. natn. Acad. Sci. U.S.A.* **90**, 4907-4911 (1993).
10. Fuchs, R. P. P. & Seeberg, E. *EMBO J.* **3**, 757-760 (1984).
11. Sancar, A., Franklin, A., Sancar, G. B. & Tang, M. S. *J. molec. Biol.* **184**, 725-734 (1985).
12. Hansson, J., Munn, M., Rupp, W. D., Kahn, R. & Wood, R. D. *J. biol. Chem.* **264**, 21788-21792 (1989).
13. Szymkowski, D. E., Yarema, K., Essigman, J. M., Lippard, S. J. & Wood, R. D. *Proc. natn. Acad. Sci. U.S.A.* **89**, 10772-10776 (1992).
14. Sweder, K. E. & Hanawalt, P. C. *J. biol. Chem.* **269**, 1852-1857 (1994).
15. Guzman, S. N. et al. *Nature* **367**, 91-94 (1994).
16. Qui, H., Park, E., Prakash, L. & Prakash, S. *Genes Dev.* **7**, 2161-2171 (1993).
17. Gileadi, O., Feaver, W. J. & Kornberg, R. D. *Science* **257**, 1389-1392 (1992).
18. Yoon, H., Miller, S. P., Pabich, E. K. & Donahue, T. F. *Genes Dev.* **6**, 2463-2477 (1992).

19. Terleth, C., van de Putte, P. & Brouwer, J. *Mutagenesis* **6**, 103–111 (1991).
20. Hanawalt, P. & Mellon, I. *Curr. Biol.* **3**, 67–69 (1993).
21. Downes, C. S., Ryan, A. J. & Johnson, R. T. *BioEssays* **15**, 209–216 (1993).
22. Wang, Z., Wu, X. & Friedberg, E. C. *Biochemistry* **31**, 3694–3702 (1992).
23. Naumovski, L. & Friedberg, E. C. *Molec. cell. Biol.* **6**, 1218–1227 (1986).
24. Morrison, A. et al. *J. Bact.* **171**, 5659–5667 (1989).
25. Wang, Z., Wu, X. & Friedberg, E. C. *J. biol. Chem.* **266**, 22472–22478 (1991).
26. Naegeli, H., Bardwell, L. & Friedberg, E. C. *J. biol. Chem.* **267**, 392–398 (1992).
27. Bardwell, L. et al. *Proc. natn. Acad. Sci. U.S.A.* (in the press).

ACKNOWLEDGEMENTS. We thank T. Donahue for the yeast strain KG119, C. Lawrence for the yeast strain CL1265-7C, H. Naegeli for purified Rad3 protein, and our colleagues for discussions and for reviewing the manuscript. Supported by grants from the US Public Health Service (E.C.F. and R.D.K.).

Stereoselectivity of DNA catenane fusion by resolvase

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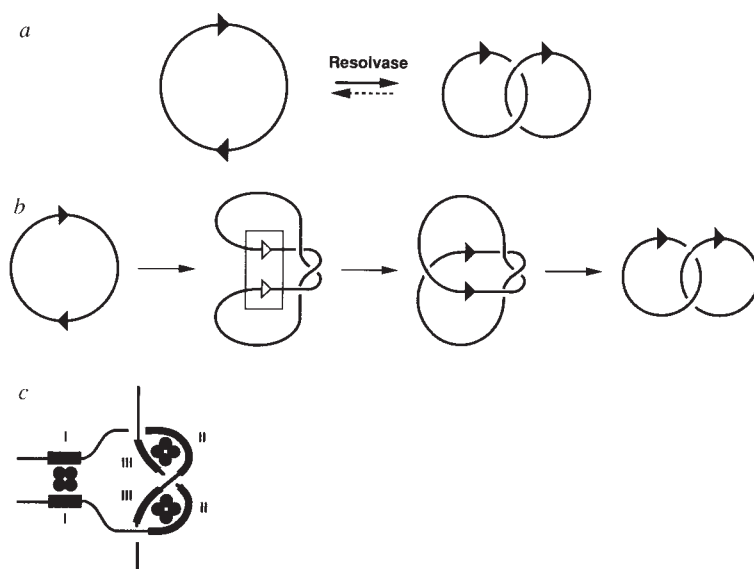
COMMUNICATIONS between distant sites on DNA often depend on the way in which the sites are connected^{1,2}. For example, site-specific recombination catalysed by Tn3 resolvase is most efficient when the 114-base-pair *res* recombination sites are directly repeated in the same DNA molecule³. *In vitro* a supercoiled plasmid substrate containing two directly repeated *res* sites gives a resolution product in which the two recombinant circles are topologically linked as a simple (two-noded) catenane (Fig. 1a). Resolvase is highly selective in forming this product rather than unlinked circles or more complex catenanes. It does not catalyse recombination between sites on separate supercoiled molecules, or between inverted sites in the same supercoiled molecule^{3–5}. Tn3 resolution removes four negative supercoils from the substrate, an energetically favourable change which may drive the reaction⁶: in relaxed or nicked circular substrates, resolution is incomplete and slower. Resolvase can catalyse fusion of the circles of a nicked or relaxed catenane, giving a single unknotted circular product^{6,7}. The fusion is the precise topological reversal of resolution, introducing four negative supercoils into a relaxed catenane substrate⁶, and should therefore not proceed if the catenane is already negatively super-

coiled. Here we study recombination between *res* sites in non-supercoiled DNA circles linked into simple catenanes. We used (+2) and (−2) catenanes, which differ only in the direction in which one circle is threaded through the other (Fig. 2a). Although stereoselectivity is a feature of enzyme catalysis, it is not obvious how resolvase can distinguish between these subtly different catenane diastereomers. A model for the intertwining of the *res* site DNA in the catalytically active complex^{4,7} predicts that only the (−2) catenane will recombine, giving unknotted and 4-noded knot circular products. We have confirmed this prediction for the Tn3 and Tn21 resolvases.

The sign of the topological nodes in the catenane resolution product was shown by electron microscopy to be negative⁸. The product topology is consistent with the pathway outlined in Fig. 1b, where in an intermediate synaptic structure, three negative interdomainal nodes are trapped when the sites of strand exchange are aligned. This scheme accounts for all the topological data on the normal reaction, as well as for minor reaction products presumed to be made by iteration of the strand-exchange mechanism within the same intermediate, and for products of reactions of substrates with mutated *res* sites^{6,8–11}.

The exclusive formation of (−2) catenane resolution product is a peculiar feature of the reaction. Although the topology of the productive synapse is established (Fig. 1b), it is not clear why it should be uniquely specified, because random collision of two *res* sites in a supercoiled molecule might trap variable numbers of interdomainal supercoils, leading to alternative product topologies. Reactions catalysed by λ Int, FLP, and Cre recombinases give products consistent with random collision^{12–15}. A 'topological filter' model for the selectivity of resolvase^{7,16} proposes that there is no selection against 'random collision' synapsis *per se*, but in a resolution substrate only one initial synapse topology (with no interdomainal supercoil nodes) is convertible into a productive synapse, in which strand exchange can occur. The conversion involves interwrapping of the two *res* sites to form a protein–DNA complex as shown in Fig. 1c. A requirement for this intermediate provides a rationale for the productive synapse topology observed (Fig. 1b). Interwrapping of the sites when any interdomainal nodes are present causes tangling of the DNA which is predicted to be energetically unfavourable⁷. A feature of the model is that at least the earlier stages of synapse formation are reversible, so that sites synapsed with trapped interdomainal nodes can dissociate and/or rearrange into a productive configuration. A structure for the productive synapse and a pathway for its formation have been proposed^{7,16}.

FIG. 1 Topological features of Tn3 resolution. Double-stranded DNA is represented as a single line, and the *res* recombination sites by black arrowheads. a, The resolution reaction of a circular DNA molecule with directly repeated *res* sites. 'Catenane fusion' is the reverse process. b, Deduced topology of synapsis and strand exchange^{6,9}. Three interdomainal supercoil nodes are trapped when the crossover points in subsite I of *res* (open triangles) are aligned in the synapse (rectangular box). Strand exchange has a right-handed sense ($X_r = +1$) (ref. 6). A proposed mechanism for strand exchange⁶ involves simultaneous cleavage of all four strands in the synapsed pair of sites, and 'simple rotation' of one pair of ends relative to the other. c, Structural model for the 2 *res* synaptic complex, as proposed for the 'topological filter' model for selectivity (see text)^{7,16}. The three resolvase-binding subsites of *res* are shown as thick black lines. In a circular substrate with directly repeated sites (compare b), formation of this structure after an initial collision that does not trap any supercoils, followed by strand exchange ($X_r = +1$), creates the observed two-noded catenane product. Resolvase molecules (black circles), shown binding to *res* as dimers, and interacting to form tetramers in the synapse, have been included in the diagram. The actual resolvase–resolvase and resolvase–DNA interactions in the synapse are not known.



The node sign of a simple catenane (-2 or $+2$; Fig. 2a) is only meaningful if the DNA in each circle can be assigned a 'direction', for example by the sequence of a recombination site. A catenane that is $(+2)$ according to the direction of a *res* site in each circle does not differ from the (-2) equivalent in sequence or geometry, only in the topological relationship of the two sites. However, if the complex shown in Fig. 1c is an obligatory intermediate in recombination, the products (if any) of catenane fusion by resolvase will be very different for $(+2)$ and (-2) catenanes (Fig. 2b). The (-2) catenane (nicked or relaxed) can yield either an unknotted circle by left-handed strand-exchange rotation; that is, by reversal of the resolution mechanism ($X_r = -1$; Fig. 1b), or a four-noded knot by right-handed rotation ($X_r = +1$). But formation of a synapse of the proposed geometry from a $(+2)$ catenane produces a tangle, and either right- or left-handed strand exchange would give an eight-noded knot. Formation of the synapse and/or the knotted products is likely to be energetically unfavourable, so that recombination of the nicked $(+2)$ catenane might be blocked.

We tested this prediction of the topological filter model in two ways. One method involved substrates containing sites for both Tn3 and Tn21 resolvases. Tn21 resolvase has amino-acid sequence homology with Tn3 resolvase, and the organization of the resolvase-binding sequences in its recombination site is very similar to that of Tn3 *res*. The two recombinases act only on their cognate sites¹⁷. Tn21 resolution *in vitro* gives a two-noded catenane¹⁸; we show here (see later) that the nodes are of the same sign as for the Tn3 product, namely negative. By constructing plasmid substrates containing two copies of each type of *res* site, catenanes could be made that were (-2) with respect to the sites of the resolvase that created them, and (-2) or $(+2)$ with respect to the other two sites (Fig. 3). These catenanes were nicked and purified, and each was treated with Tn3 or Tn21 resolvase (Fig. 4). The resolvases acted on their own sites when they were in the (-2) relationship, to give fusion products. The major products were unknotted circles, but small amounts of four-noded molecules were formed also, as expected. Restriction

enzyme digestion showed that the fusion products were of the same size as the original plasmid substrate (Fig. 4), and further digests (not shown) proved that no unexpected DNA rearrangement had taken place. No recombination products were observed when the resolvases were presented with catenanes containing their sites in the $(+2)$ relationship.

The behaviour of Tn3 resolvase on catenanes made by Tn21 resolvase (and vice versa) demonstrated that the normal Tn21 resolution product also has exclusively (-2) topology; for example, the catenane produced by Tn21 resolvase from pCP10 (in which all the sites are directly repeated) was fused by both Tn3 and Tn21 resolvases, and must therefore be (-2) with respect to both pairs of sites. Likewise, both resolvases failed to recombine $(+2)$ catenated sites. We infer that Tn21 resolution proceeds via a synapse with the same topology as that of the Tn3 reaction.

A related experiment used a plasmid (pMA19) that has three Tn3 *res* sites, one of which is inverted with respect to the other two. This plasmid was resolved quantitatively to give a two-noded catenane; one circle of the catenane contains a single *res*, and the other contains two *res* sites that are in inverted repeat (Fig. 3). The catenane nodes can be designated as $(-)$ with respect to the directions of *res* sites A and B, or $(+)$ with respect to sites A and C. Recombination between sites A and B in the nicked catenane was predicted to give unknotted circle fusion product, whereas recombination between A and C, if observed, should give an eight-noded knot. When pMA19 was treated with Tn3 resolvase (Fig. 4c), the major fusion product was unknotted circle; no eight-noded knot was detected. Restriction enzyme

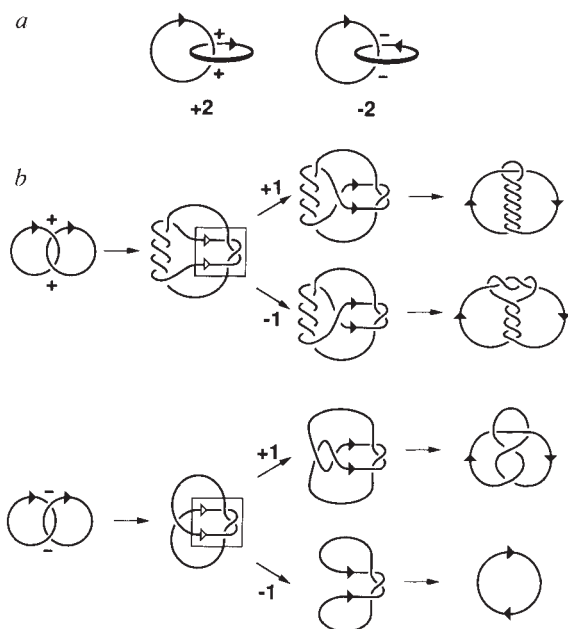


FIG. 2 Catenane topology. a, Left, $(+2)$, and right, (-2) catenanes. The direction of the DNA in each circle is defined by the sequence of a *res* site (black arrowhead). The only difference between the two structures is the orientation of one of the *res* sites. b, Synapsis and strand exchange as in the model shown in Fig. 1b and c, for $(+2)$ (top) and (-2) (bottom) catenanes. The complex topology of the productive synapse formed from the $(+2)$ catenane is predicted to be energetically unfavourable. For each synapse, the products of right-handed ($X_r = +1$) and left-handed ($X_r = -1$) strand-exchange rotation are shown.

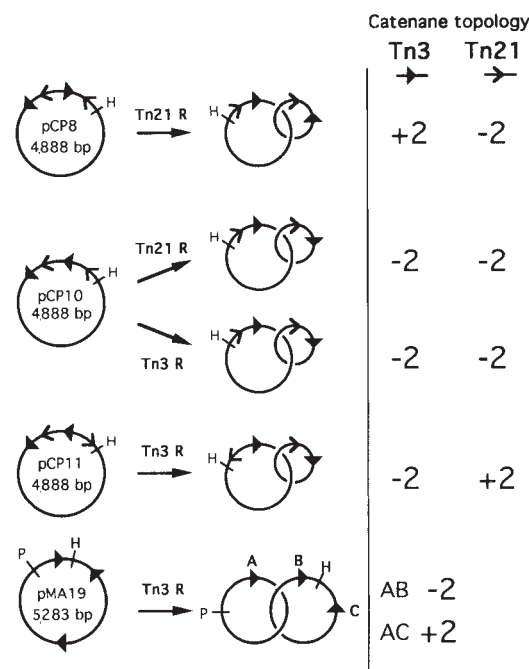


FIG. 3 Substrates for catenane experiments. Plasmids pCP8, pCP10, pCP11, and pMA19, and their resolution production catenanes. Tn3 *res* sites are represented as filled arrowheads and Tn21 *res* sites as arrowheads. R, resolvase; P, *Pst*I restriction site; H, *Hind*III restriction site. The three Tn3 *res* sites in the catenane from pMA19 are labelled A, B and C (referred to in the text and in the legend to Fig. 4).

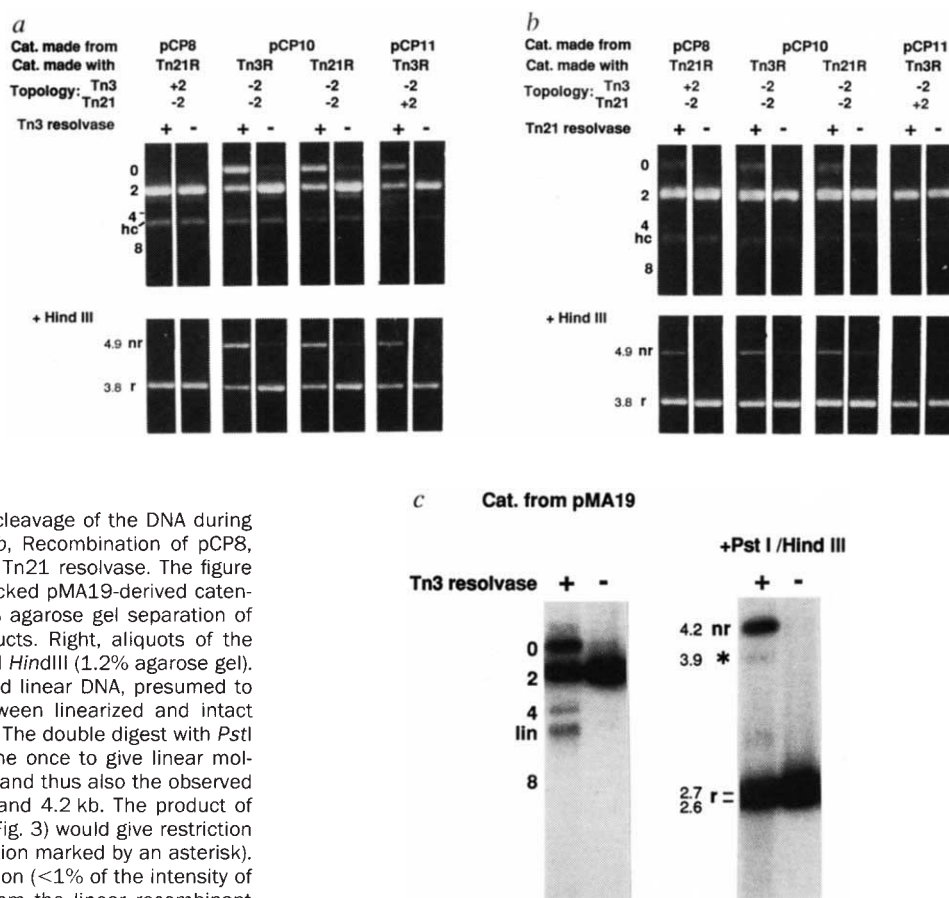
METHODS. pCP8 and pCP10 have been described¹⁷. pCP11 is like pCP10, except that a *Pst*I-*Pst*I fragment containing Tn21 *res* is inverted. pMA19 is a derivative of pBR322, with fragments containing Tn3 *res* inserted into the *Eco*RI, *Pvu*II and *Bam*HI sites. Details of its construction will be given elsewhere. Plasmid DNA was purified by caesium chloride gradient ultracentrifugation. Resolution and nicking with DNase I have been described^{6,10}. Nicked catenanes were purified by excision of the appropriate bands from low-melting-point agarose gels ('Seaplaque'; Flowgen), followed by extraction of the agarose with phenol and chloroform, and ethanol precipitation.

FIG. 4 Recombination of nicked catenanes by Tn3 and Tn21 resolvases. **a**, Recombination of pCP8, pCP10 and pCP11-derived catenanes (Cat.) (Fig. 3) with Tn3 resolvase. Top, 0.7% agarose gel separation of nicked catenanes and recombination products. Bottom, aliquots of the same samples after digestion with *Hind*III. (1.2% agarose gel). *Hind*III cuts at a unique site in each molecule, linearizing the larger (3.8 kb) circle of the catenane (r), or the plasmid-sized (4.7 kb) fusion product (nr). nc, nicked circle; the numbers 2,4,8 indicate position of species of the given number of topological nodes, assigned by comparison with a reference mixture of DNA knots made from pCP11 with topoisomerase II¹⁰; hc, the 3.8-kb circle component of the catenane (nicked or linear), freed from the other circle by some nonspecific cleavage of the DNA during purification from agarose (Fig. 3 legend). **b**, Recombination of pCP8, pCP10 and pCP11-derived catenanes with Tn21 resolvase. The figure is arranged as in **a**. **c**, Recombination of nicked pMA19-derived catenane (Fig. 3) with Tn3 resolvase. Left, 1.2% agarose gel separation of nicked catenane and recombination products. Right, aliquots of the same samples, after digestion with *Pst*I and *Hind*III (1.2% agarose gel). Labels are as in **a**; lin, 5.3-kb plasmid-sized linear DNA, presumed to be made by intermolecular reactions between linearized and intact circles from the catenane (see legend to **a**). The double digest with *Pst*I and *Hind*III cuts each circle of the catenane once to give linear molecules of 2.6 and 2.7 kb, and cuts pMA19 (and thus also the observed fusion product) into two fragments of 1.1 and 4.2 kb. The product of recombination between *res* sites A and C (Fig. 3) would give restriction fragments of 1.4 kb and 3.9 kb (at the position marked by an asterisk). The faint band seen at the asterisked position (<1% of the intensity of the band at 4.2 kb) is probably derived from the linear recombinant DNA in the undigested reaction products.

METHODS. Purified DNA catenanes were treated with either Tn3 or Tn21 resolvase. These reactions were carried out as described¹⁰ for 20 h at 37 °C in buffer containing 50 mM Tris-HCl, pH 8.2, 50 mM NaCl (for Tn3 resolvase), 100 mM NaCl (for Tn21 resolvase), 5 mM spermidine, 5 mM MgCl₂, 0.1 mM EDTA, 20% w/v glycerol. Agarose gels were run, stained with ethidium bromide, and photographed, as described¹⁰.

digests showed that recombination was only between sites A and B (Fig. 4c), and that no other rearrangements had taken place (data not shown). Recombination is thus specific to the pair of sites in the (-2) catenane relationship.

Previous experiments on the topological selectivity of Tn3 resolvase¹¹ and Mu transposition¹⁹ used multiply-interlinked supercoiled catenanes as substrates. In four-noded and more complex catenanes, the axis of at least one of the DNA circles is necessarily distorted from planar, that is, it has writhe²⁰, and resolvase could potentially sense the relationship of two *res* sites on the circles directly, by the way that they were intertwined. However, in the nicked two-noded catenanes used here, there is



Gels were also blotted onto Hybond N membrane (Amersham) by a standard neutral transfer method, then probed with random-primed ³²P-labelled DNA to detect weak bands: **a** and **b** are ethidium-stained gels, and **c** is an autoradiograph. Long exposure of the blotted gels in **a** and **b** did not detect any bands attributable to intramolecular recombination of the (+2) catenanes (not shown).

no supercoiling and no writhe²⁰. We propose that the nature of the selection is a thermodynamic barrier to formation of a productive synapse of the required geometry in the (+2) catenane, because of the necessary tangling of the DNA (Fig. 2b). The prohibition of resolvase-catalysed recombination between *res* sites on separate circular molecules, or between sites in inverted repeat, can be accounted for in a similar way, because productive synapse formation would similarly require DNA entanglement. The specific formation of (-2) catenane resolution product is explained because initial encounters of *res* sites that would potentially give other products do not lead to productive synapses, for the same reasons^{7,16}. □

Received 20 September; accepted 17 December 1993.

- Gellert, M. & Nash, H. A. *Nature*, **325**, 401-404 (1987).
- Wang, J. C. & Gaeber, G. N. *Science*, **240**, 300-304 (1988).
- Hatfull, G. F. & Grindley, N. D. F. in *Genetic Recombination* (eds Kucherlapati, R. & Smith, G. R.) 357-396 (Am. Soc. Microbiol., Washington DC, 1988).
- Stark, W. M., Boocock, M. R. & Sherratt, D. J. *Trends Genet.*, **5**, 304-309 (1989).
- Reed, R. R. *Cell*, **25**, 713-719 (1981).
- Stark, W. M., Sherratt, D. J. & Boocock, M. R. *Cell*, **58**, 779-790 (1989).
- Boocock, M. R., Brown, J. L. & Sherratt, D. J. *UCLA Symp. molec. cell. Biol.*, **47**, 703-718 (1987).
- Wasserman, S. A. & Cozzarelli, N. R. *Proc. natn. Acad. Sci. U.S.A.*, **82**, 1079-1083 (1985).
- Wasserman, S. A., Dungan, J. M. & Cozzarelli, N. R. *Science*, **229**, 171-174 (1985).
- Stark, W. M., Grindley, N. D. F., Hatfull, G. F. & Boocock, M. R. *EMBO J.*, **10**, 3541-3548 (1991).

- Benjamin, H. W. & Cozzarelli, N. R. *J. biol. Chem.*, **165**, 6441-6447 (1990).
- Mizuuchi, K., Gellert, M., Weisberg, R. A. & Nash, H. A. *J. molec. Biol.*, **141**, 485-494 (1980).
- Pollock, T. J. & Nash, H. A. *J. molec. Biol.*, **170**, 1-18 (1983).
- Beatty, L. G., Babineau-Clary, D., Hogrefe, C. & Sadowski, P. D. *J. molec. Biol.*, **188**, 529-544 (1986).
- Abremski, K. & Hoess, R. H. *J. molec. Biol.*, **184**, 211-220 (1985).
- Boocock, M. R., Brown, J. L. & Sherratt, D. J. *Biochem. Soc. Trans.*, **14**, 214-216 (1986).
- Parker, C. N. & Halford, S. E. *Cell*, **66**, 781-791 (1991).
- Castell, S. E., Jordan, S. L. & Halford, S. E. *Nucleic Acids Res.*, **14**, 7213-7226 (1986).
- Craigie, R. & Mizuuchi, K. *Cell*, **45**, 793-800 (1986).
- Wasserman, S. A., White, J. H. & Cozzarelli, N. R. *Nature*, **334**, 448-450 (1988).

ACKNOWLEDGEMENTS. We thank our colleagues in Glasgow and Bristol who contributed to this work. The research was supported by the Royal Society, the Wellcome Trust, and the SERC.

Protein and peptide paraphernalia

Microwell plates with covalently bound nickel chelate, a robotic crystallization system for experiments using hanging drop and sitting drop methods, and 10-mm probes for biomolecular applications are highlighted this week.

XENOPORE's new **microwell plates with a nickel chelate** covalently attached provide a new support surface for peptide assays (*Reader Service No. 100*). This nickel chelate specifically binds sequences of histidine units. The company says that any peptide or protein which contains or which can be tagged with a sequence of four or five histidine units in a row will bind specifically to the chelate. An ELISA test can then be constructed on the chelate assay plate, using the immobilized peptide as the capture probe. The plates are said to be particularly useful for screening antibodies against peptides or protein fragments. They are available in clear plates for colorimetric assays, and in opaque white and black versions for chemiluminescent and fluorescent assays.

Procise, the new **protein sequencing system** from Perkin-Elmer's Applied Biosystems Division, offers programmability and control using a Macintosh Quadra, either gas or pulsed-liquid chemistries, multiple cartridge configurations, and extra bottles to accommodate future advances in the chemistry (*Reader Service No. 101*). Up to four reaction cartridges enable sequential

Oxford Molecular's new version of its **Iditis protein structure database** incorporates an intuitive graphical user interface (GUI) and an improved search engine (*Reader Service No. 102*). The new X-Motif GUI is said to be a considerable improvement over the original SQL (structured query language) interface. In addition, the Iditis search engine has been rewritten to deliver significant increases in search speeds. The company says that the GUI reduces the query-building process to a selection of onscreen 'buttons', which reflect the types of searches commonly made on the database. Users are guided to the desired query through a series of 'intelligent' dialogue boxes. Query conditions are set up using pull-down menus containing the relevant options, and once all conditions have been entered Iditis builds the necessary SQL query, to return the motifs from the database. Iditis contains a large collection of pre-calculated data derived from the Brookhaven DataBank (PDB). The number of structures already deposited in the PDB already exceeds 900, with more than 50 new structures added every month.

■ Crystallization

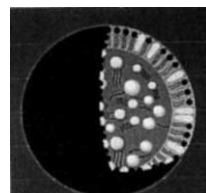
Crystallographers may be interested in the new **robotic protein crystallography system** from ICN Biomedicals which is designed to increase experimental precision and accuracy for protein crystallization experiments that use hanging drop and sitting drop techniques (*Reader Service No. 103*). Liquid level sensing which automatically determines the surface level of protein solution, the ability to conduct 16 crystallization experiments in a 4 × 4 array and the ability to select up to six different solutions using an eight-port rotary valve are just a few of the system's design features. Preparation of the

conditions for protein crystal growth are found by optimizing the concentrations of each solution used in the grid.

Hampton Research has developed a new **silica hydrogel kit for growing macromolecular crystals**, which is available from Stratech Scientific (*Reader Service No. 104*). Silica gels are said to have several advantages: they are stable, can be used over a wide temperature range (0–60 °C), and are compatible with a variety of precipitants and additives used in the growth of crystals. The kit is supplied with all the materials needed to make gels at room temperature which polymerize rapidly and form a porous matrix with a pH of 7.0. The silica hydrogel can be used for liquid gel, liquid-gel-liquid and vapour diffusions, as well as dialysis crystallization methods.

■ Reagents and services

Human recombinant apolipoproteins E2, E3 and E4, now available from PanVera, are produced by baculovirus-mediated expression in the *Spodoptera frugiperda* insect cell line (*Reader Service No. 105*). Apo E is purified by affinity chromatography and supplied either in solution or lyophilized. The discovery by Corder *et al.* (*Science* **261**, 921; 1993) that apolipoprotein E (apo E) isoforms are associated with the onset of Alzheimer's disease in late-onset families has renewed interest in the function of this member of the apolipoprotein family. Specifically, these researchers showed that there is a prevalence of Alzheimer's in individuals with the Apo E4 isoform. In the prevailing hypothesis, Apo E4 is said to promote the multimerization of β -amyloid peptide plaque formation in neuronal tissues, ultimately leading to nerve degeneration. A less widely held view focuses on the ability of Apo E3 and E2 to stabilize the neuronal microtubule protein *tau* and thereby prevent nerve cell death.



PanVera's human recombinant apolipoproteins.



ABI's Procise protein sequencing system offers gas or pulsed-liquid chemistries and multiple cartridge configurations.

sequencing. Individual cartridges can be independently programmed to run gas-phase while another can be programmed for pulsed-liquid chemistry. Multiple samples can be loaded for 24-h operation, seven days a week. The system also continually monitors temperature and pressure changes. Eleven optical sensors detect the delivery of fluid to ensure that reagents have been delivered and that the sample has been exposed to the correct amount of reagents. The system accommodates all the most popular sample supports and sequencing methods. Every aspect of the system's operation — from set-up through to analysis — is directed from the Macintosh. The Procise system is available in a range of cartridge configurations and includes the Macintosh Quadra computer and standalone PTH HPLC system.



ICN's robotic crystallization II system.

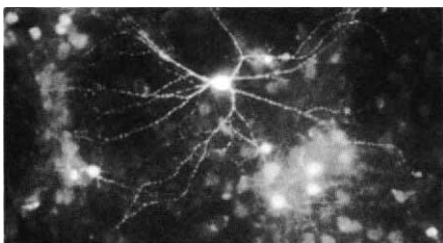
experiments can be achieved in either two multichamber vapour diffusion plates: a Linbro Plate which uses coverslips from which the droplet hangs when transferred, or a Cryschem Plate which uses a post in the centre of each well upon which the droplet sits when transferred. ICN says that the best

The EGGstract **IgY purification system** from Promega provides an alternative method of isolating immunoglobulins (IgY antibodies) from egg yolks (*Reader Service No. 106*). Promega says that yields are 90–100 mg of IgY per egg yolk, and that greater than 75 per cent pure product can be obtained in less than an hour.

Amersham's drug development service has launched a new assay that can be used for **screening potential AIDS drugs** (*Reader Service No. 107*). The new assay, part of the company's range of scintillation proximity assay (SPA)-based screening assays, is designed to identify inhibitors of the enzyme HIV-1 integrase. This enzyme is required for efficient viral gene expression and is essential to the infective nature of the virus. It therefore represents an important therapeutic target for potential anti-HIV drugs. The SPA format lends itself to automation for high-volume drug screening. Amersham also markets several other assays for HIV proteinase, reverse transcriptase and RHase H, which can be used for secondary screening purposes in combination with the HIV-1 integrase assay.

The Institut für Retrovirale is now offering a **retroviral screening service** to academic and industrial laboratories (*Reader Service No. 108*). The service is directed towards biological safety and quality control, as well as bioprocess monitoring by the detection of virus contamination in cell cultures, biopharmaceutical products and biological samples. The institute also provides an anti-retroviral drug testing service. The results obtained are confidential and non-use of the experimental results is guaranteed.

Calretinin is a neuron-specific calcium binding protein found in the central nervous system and the retina. It is present in subsets of neurons throughout the brain and spinal cord, including sensory ganglia, and it shares about 55 per cent amino acid sequence with calbindin, another calcium binding protein found in the brain and periphery. Preliminary studies seem to indicate that calretinin, like calbindin, may have some resistance to excitotoxicity. Biochemical



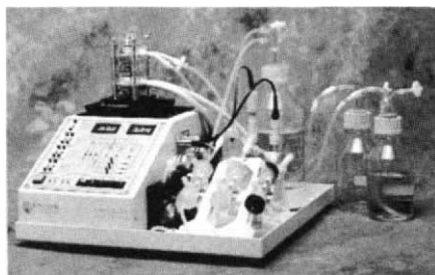
Chemicon's polyclonal antibody to calretinin — a neuron-specific calcium binding protein.

studies have shown that calretinin inhibits the phosphorylation of a 39 kilodalton mitochondrial membrane protein. Chemicon is now offering a **polyclonal antibody to calretinin**, which is said to have been used successfully on the brain of human, rat, monkey, guinea pig, cow, mouse and chick by both fluorescence and immunoperoxidase techniques (*Reader Service No. 109*). The antibody is also intended for western blotting.

RIM-1 (rhodamine-conjugated) and FIM-1 (fluorescein-conjugated), two new **fluorescent probes for protein kinase C** (PKC) that are available from Kamiya Biomedical, are fluorescent derivatives of the bisindolylmaleimide inhibitors of PKC (*Reader Service No. 110*). The company says that RIM-1 and FIM-1 retain much of the specificity and potency of the parental compound and target the ATP-competitive catalytic domain of PKC. Consequently, they can serve as rapid and simple cytological probes for visualizing PKC *in vitro*. FIM-1 diacetate is permeable to living cells and can be used for studies of PKC *in vivo*, although Kamiya Biomedical points out that its effect on PKC has not been clearly defined. RIM-1 is impermeable to living cells.

Cell culture

Unisyn Technologies says that its Cell-Pharm System 1000 is intended as a research-to-pilot scale **cell culture system** for producing biomolecules, providing concentrated, debris-free supernatants, simplifying downstream purification and improving yields



Unisyn's benchtop bioreactor.

(*Reader Service No. 111*). Using Unisyn's hollow-fibre bioreactor technology, the system is capable of producing from 200 to 3,000 mg per month, depending upon whether one, two or three Cell-Pharm bioreactors are used. The system comes in a self-contained benchtop unit that does not require an incubator, and operates with a dual-head peristaltic pump. It features a built-in oxygen delivery system and automated pH control. The front panel controls, flow diagram and LED provide the operator with up-to-date information on the status of the system.

BioWhittaker's new **serum-free medium**, UltraCHO, supports the growth of Chinese Hamster Ovary (CHO) cells and their recombinant derivatives (*Reader Service No. 112*). UltraCHO is made up of a modified DMEM:F12 base which is optimized to support the growth of transfected and nontransfected CHO cells. The formulation is supplemented with insulin, transferrin and purified proteins — the final product containing less than 300 µg ml⁻¹ protein. Eliminating serum from the growth environment is said to enable a more thorough analysis of raw material, thus increasing control over the presence of adventitious agents and decreasing the lot-to-lot variation associated

with serum products. Furthermore, as serum-free formulations are generally less complex and contain lower amounts of protein, downstream purification of recombinant proteins should be simplified. UltraCHO has been used to establish cultures of CHO-PA and CHO-ST4.2 in hollow-fibre bioreactors, supporting the production of plasminogen activator and CD4, respectively.



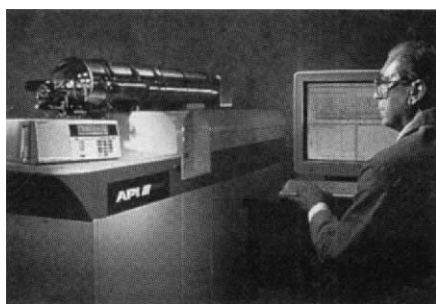
CHO cell medium.

Hardware

Varian's new 10-mm probe is designed to enable **nuclear magnetic resonance** (NMR) spectroscopy to be used in the study of very large biological molecules (*Reader Service No. 113*). In the past, NMR analysis of many enzymes, proteins and other large biological molecules has been difficult using this method due to their limited solubility, which reduces sensitivity, and their tendency to aggregate in the concentrated solution required for a 5-mm sample analysis. These new probes are said to eliminate these limitations by enabling NMR analysis on very dilute 10-mm samples.

Vestec introduces its new automated benchtop reflector matrix-assisted laser desorption **time-of-flight mass spectrometer** for determining molecular weights on biopolymers (*Reader Service No. 114*). In addition to high-resolution molecular weight information on peptide mixtures under 12,000 daltons, Vestec says that applications have been extended to the study of metastable decompositions. Spectra obtained with this configuration may provide sequence information on proteins/peptides directly without the use of chemical modification or digestion. The instrument has been designed for rapid sample throughput and routine use. The LaserTec line uses a video monitor to view each sample and a joystick for fast adjustment of sample position on a 100-sample plate on an x-y stage. The BenchTop II can also operate unattended. It is supplied in a benchtop cabinet and is provided with a 1.2 m vertical linear flight tube and a 2.6 m reflector.

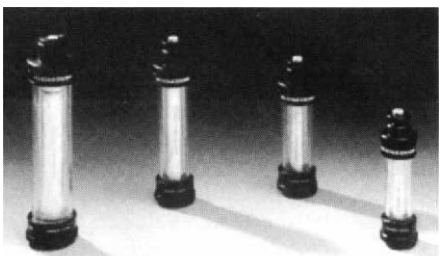
The API IIIplus LC/MS/MS system from Perkin-Elmer Sciex is an **atmospheric pressure ionization mass spectrometer** for use in biotechnology, pharmaceutical and environmental applications (*Reader Service No. 115*). The system accepts the PE Sciex IonSpray (pneumatically-assisted electrospray) and Heated Nebulizer-APCI (atmospheric pressure chemical ionization) inlets,



Perkin-Elmer Sciex's LC/MS/MS system.

which are intended for qualitative and quantitative bioanalytical, environmental and general analytical applications. The dynamic gas collision cell is designed significantly to increase sensitivity and mass resolution to resolve triply charged daughter ions at any mass. The system's simplified ion optics provide the direct transfer of ions without heating to the mass spectrometer, enabling determination of the molecular weight of noncovalently bound complexes. The design also features a high capacity cryogenic pumping system. The system is controlled by a Macintosh data system for data acquisition, data reduction and reporting. Software includes MacBioSpec, PeptideSeq, PeptideScan and PeptideMap.

Bio-Rad's new range of prepacked ion-exchange columns are designed for use with high-resolution chromatography systems (*Reader Service No. 116*). The Bio-Scale columns, packed with Macro-Prep ion-exchangers, can be used for the separation of proteins, peptides and polynucleotides. Bio-Rad says that the narrow particle



Bio-Scale columns from Bio-Rad.

size distribution of the 10- μ m packing, together with an optimized packing procedure, produces good resolution of biomolecules at high flow rates and with very low back pressures. The columns are also said to demonstrate low nonspecific binding of biomolecules. Four sizes are available — 2, 5, 10 and 20 ml.

New MassMap protein identification software is now available from Finnigan Mat for use with the company's Lasermat mass analyser. This set-up is said to provide protein identification in a few minutes, require only low picomole quantities of sample, and offer a rapid alternative to Edman sequencing (*Reader Service No. 117*). MassMap, a program for IBM PCs or compatible computers running Microsoft Windows

NT, identifies proteins using the masses of fragments generated by proteolytic digest and determined using Lasermat to search a database of over 140,000 protein sequences. The database used is that distributed by the National Center for Biotechnology Information (NCBI) and incorporates sequences drawn from GenBank, EMBL, SwissProt, PIR and several other smaller databases. Standard amino acid masses and cleavage rules for most common proteolytic enzymes are predefined in the MassMap software, which calculates the fragment molecular weights for complete digests with a range of proteolytic reagents for each of the sequences in the database. Users can, however, define alternative mass values for specific applications. MassMap identifies a protein by searching the NCBI database for likely matches to the peptide mass fingerprint. Each sequence is scored in order to indicate the degree of correspondence between the mass fingerprint calculated for the database protein and the experimentally determined mass fingerprint for the sample protein. The use of multiple search windows makes for easy comparison of matches where a single protein is digested separately with a variety of enzymes.

■ Peptides

Many new bioactive peptides have been added to American Peptide's 1994-95 catalogue (*Reader Service No. 118*). The company is offering a complete range of peptides, such as endothelins, including new ET-B receptor antagonist, beta amyloids, cholecystokinin-related peptides, pituitary adenylate cyclase activating polypeptides, neuropeptides, calcitonins and calcitonin gene-related peptides. The catalogue also includes resins, amino acids and reagents for peptide synthesis. American Peptide has also expanded its production facility for bulk and cGMP synthesis, and is specialized in custom synthesis of peptides, containing both natural and unnatural amino acids.

The APC 522 automated peptide cleaver from Abimed automates the steps involved in cleavage and work-up of up to 30 synthetic peptides (*Reader Service No. 119*). The system uses a standard TFA cleavage protocol utilizing tert-butyl scavengers. The work-up scale ranges from 10-50 mmol and the stated yield is 70 per cent or more of crude peptide. For protection, the system is completely covered by a hood and is flushed with nitrogen. A ventilation system is used to remove the pungent smell of scavengers. Operation of the instrument is via a controller with an 8-line display and 3.5-inch floppy disk drive. An optimized chemistry protocol with a minimum of user-defined parameters is delivered as a default. □

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Feeling the groove

Variation in ways in which proteins can identify and lock on to genes due for transcription is illuminated by studies of the Arc repressor, which has a β -sheet, rather than α -helical, recognition motif.

CENTRAL to any data storage system is the ability to access and retrieve information. Each block of data must be identified to distinguish it from the other material in the data base. The nature of the retrieval system is then determined by the data storage medium. The information stored in a genome, in the form of gene sequences, must be accessed, often in a highly selective manner, and converted into the machinery that builds, and maintains, the organism in question.

Proteins of the transcription apparatus, the nuts and bolts of the genome information retrieval system, are able to identify the particular gene or genes that are destined to be transcribed into RNA, which is then frequently translated into protein. New studies^{1,2} of one such transcription factor, the Arc repressor of *Salmonella* bacteriophage P22, provide an illustration of the means by which a protein can identify and get to grips with the short DNA sequence that flags the presence of a particular gene.

Consideration of the structure of DNA indicates that there is enough information provided by the hydrogen bond donors and acceptors on the exposed edges of the bases in the major, but not the minor, groove to distinguish each of the four bases in DNA³. Indeed, most sequence-specific recognition of DNA is through the major groove.

What kind of protein motifs 'feel the groove'? Early studies noted that a two-stranded anti-parallel β -sheet has features that are complementary to those of both double-stranded RNA³ and DNA⁴ and such peptides fit comfortably into the minor and major grooves of DNA⁴⁻⁶. In contrast the first structures of sequence-specific DNA-binding proteins, all prokaryotic regulators, effected sequence recognition in the major groove by a so-called recognition α -helix⁷.

To date, most of the known sequence-specific interactions with DNA are

mediated by residues on α -helices. These α -helices are presented to the major groove in the context of a variety of structural elements (the helix-turn-helix, the basic-helix-loop-helix and so on)⁷. Nonetheless β -sheet motifs are also used in proteins that interact with both the major and, surprisingly, minor grooves.

The Arc repressor is a member of the ribbon-helix-helix family of transcription factors, which also includes the MetJ repressor⁸. Both proteins bind to their cognate operator as tetramers, consisting of a dimer of dimers. Each monomer provides one of the ribbons of the recognition β -sheet and each dimer interacts with an operator 'half-site', TAGA in the case of Arc. Although individual Arc dimers display two-fold symmetry, the operator half-site to which they bind is asymmetrical. Inevitably, symmetry-related amino acids must interact with non-symmetry-related base-pairs; Asn 11' on one β -ribbon interacts with T(A:T)GA in the TAGA box whereas Asn 11 on the other β -strand makes a contact with TA(G:C)A. It seems likely that the flexibility of the amino-acid side chains at the interface facilitate this asymmetrical interaction.

The different DNA contacts mediated by equivalent residues in the Arc dimer illustrates the scope for variability in protein-DNA interactions and again emphasizes the difficulties of trying to decipher any type of detailed code for protein-DNA interactions.

An important part of the recognition process seems to involve a conformational adjustment of the β -sheet on binding; two phenylalanines are unpacked from the hydrophobic core and are inserted between phosphate oxygens of the DNA backbone, for example. Overall, the interaction of the Arc dimer with the phosphate backbone lining the edges of the major groove and flanking the β -ribbon is symmetrical. Such interactions are likely to lock the β -sheet tightly into the major groove, preventing wobbling or twisting of the sheet.

The mutagenesis experiments emphasize that although the recognition sheet furnishes the base-specific contacts, effective sequence recognition depends on a range of different and not necessarily direct interactions between DNA and protein. Base-specific interactions are essential, of course. Surprisingly, asymmetrical rather than symmetrical contacts contribute most to binding energy. But

interactions across the tetramer interface, involved in cooperativity, and linkage of β -sheet-major groove to phosphate contacts are also important for binding.

Two other classes of DNA-binding protein have been characterized that interact with DNA through β -sheet motifs. The prokaryotic HU family of proteins⁸ is thought to use a two-stranded β -sheet to interact with the minor groove, in a fashion that is reminiscent of the earlier modelling studies^{4,5}. As might be expected, given the paucity of sequence-dependent features in the minor groove, the HU protein binds DNA non-specifically. Surprisingly the IHF protein binds in sequence-specific manner. How this is achieved will have to await the structure of the protein-DNA complex.

The 'saddle and stirrups' binding surface of the TATA-box binding protein TBP, which consists of a 10-stranded sheet, also interacts in a sequence-specific manner with the minor groove. The structure of the complex reveals a highly unusual protein-DNA interface^{9,10}. The DNA is grossly distorted and the minor groove is splayed open, permitting a series of side-chain-base interactions that, in combination with the deformability of the run of TATA bases, determine sequence specificity.

Why is the α -helix the most prevalent recognition motif used by sequence-specific DNA-binding proteins? Is there some intrinsic advantage in using this motif, as opposed to β -sheets? Perhaps the β -sheet fulfils a different role — as in TBP? Or are the features of present-day DNA-binding proteins merely a reflection of the vagaries of evolution? At least some of these questions should be answered by the ever more detailed study of this fascinating class of proteins.

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Also in this month's *Nature Structural Biology*: folding of cytochrome *c* in the absence of intermediates; dimerization of transmembrane α -helices; two distamycin A molecules in the minor groove of B-DNA; the mechanism of allosteric activation in bacterial L-lactate dehydrogenase; substrate-channelling in dihydrofolate reductase-thymidylate synthase; and growing protein crystals on lipid layers.

1. Raumann, B.E. et al. *Nature* **367**, 754-757 (1994).
2. Brown, B.M. et al. *Nature struct. Biol.* **1**, 164-168 (1994).
3. Seeman, N.C., Rosenberg, J.M. & Rich, A. *Proc. natn. Acad. Sci. U.S.A.* **73**, 804-808 (1976).
4. Carter Jr, C.W. and Kraut, J. *Proc. natn. Acad. Sci. U.S.A.* **71**, 283-287 (1974).
5. Church, G.M., Sussman, J.L. & Kim, S.-H. *Proc. natn. Acad. Sci. U.S.A.* **74**, 1458-1462 (1977).
6. Chou, P.Y., Alder, A.J. & Fasman, G.D. *J. molec. Biol.* **96**, 29-45 (1975).
7. Pabo, C.O. & Sauer, R.T. *A. Rev. Biochem.* **61**, 1053-1095 (1992).
8. Phillips, S.E.V. *Curr. Opin. struct. Biol.* **1**, 89-98 (1991).
9. Kim, Y. et al. *Nature* **365**, 512-520 (1994).
10. Kim, J.L., Nikolov, D.B. & Burley, S.K. *Nature* **365**, 520-527 (1994).